REMARKS

Status of the Claims

Claims 1-11, 19, 22, and 23 are pending in the present application. Claim 1 has been amended to correct a typographical error. Claim 11 has been amended for purposes of clarification. Support for this amendment can be found, for example, on page 23, lines 19-29. No new matter has been added by way of this amendment.

The Examiner is respectfully requested to withdraw the rejection and allow claims 1-11, 19, 22, and 23. In any event, the Examiner is requested to enter the above amendments for purposes of furthering prosecution. These amendments were not made earlier because Applicant earnestly believes that the specification is enabling for the breadth of the claims as originally drafted. Reconsideration and reexamination is respectfully requested in view of the following remarks.

The Rejections Under 35 U.S.C. § 112, First Paragraph, Should be Withdrawn

Enablement

The Examiner rejected claims 1-11, 19 and 22-23 under 35 U.S.C. § 112, first paragraph, on the grounds that the specification does not enable one skilled in the art to make or use the invention. This rejection is respectfully traversed.

The Examiner asserts that the specification, while enabling for nucleic acids encoding SEQ ID NO:2, 4, or 6, host cells, plants, plant cells and seeds comprising them, and a method of using them to make SEQ ID NO:2, 4, or 6, does not reasonably provide enablement for methods and compositions drawn to nucleic acids encoding pesticidal proteins with 95% sequence identity to SEQ ID NO:2, 4, or 6, nucleic acids with 95% identity to SEQ ID NO:1, 3, or 5, or a complement of those nucleic acids, host cells, plants, plant cells and seeds comprising them, and a method of using them to make a pesticidal protein with 95% identity to SEQ ID NO:1, 3, or 5. The Examiner states that the specification fails to provide guidance for which amino acids of SEQ ID NO:2, 4, or 6 can be altered and to which other amino acids, and which amino acids must not be changed, to maintain the activity of the encoded protein, as well as which regions of the protein can tolerate insertions and still produce a functional protein.

The Examiner appears to be suggesting that, in order to satisfy the enablement requirement, Applicants must demonstrate that every pesticidal polypeptide and variant and fragment thereof encompassed by the claims could be used to successfully practice the invention, such that no experimentation would be required. According to the applicable case law, however, the test of enablement is not whether experimentation is necessary to make and use an invention, but rather if experimentation is necessary, whether it is undue. *In re Angstadt*, 198 USPQ 214, 219 (C.C.P.A. 1976). Furthermore, a considerable amount of experimentation is permissible if it is merely routine or if the specification provides a reasonable amount of guidance in which the experimentation should proceed. *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988).

The test of whether an invention requires undue experimentation is not based on a single factor, but rather is a conclusion reached by weighing many factors. *Id.* at 1404. Factors to be considered in determining whether undue experimentation is required include the quantity of experimentation necessary, the amount of guidance provided in the specification, the presence of working examples of the invention in the application, the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability in the art, and the breadth of the claimed invention. *Id.* Accordingly, the holding of *Wands* does not require that Applicants provide as working examples every pesticidal polypeptide that could be used to practice the present invention. Rather, *Wands* sets out factors to be considered in determining whether undue experimentation is required to make and use the invention.

The Examiner maintains that the specification does not enable one of skill in the art to make and use nucleic acids that encode polypeptides that retain pesticidal activity and have at least 95% sequence identity to SEQ ID NO:1, 3, or 5, or 95% sequence identity to a nucleotide sequence that encodes SEQ ID NO:2, 4, or 6. The Examiner incorrectly bases this conclusion solely on the number of possible nucleic acids having the recited percent identity to SEQ ID NO:1, 3, or 5, or a nucleotide sequence encoding SEQ ID NO:2, 4, or 6 while continuing to dismiss the other factors set forth in *Wands* for assessing whether undue experimentation is required. In particular, the Examiner has continued to discount the guidance provided in the specification and the working examples set forth in the application (page 6 of the Office Action mailed August 15, 2006).

First, sufficient guidance for making and using the recited sequences is present in the specification. The claimed variants and fragments of SEQ ID NO:1, 3, or 5, or nucleotide sequences encoding SEQ ID NO:2, 4, or 6 are limited by a percent identity (i.e., 95% identity) and further limited by the functional requirement that they possess pesticidal activity. Guidance for preparing variants and fragments of SEQ ID NO:1, 3, or 5, or nucleotide sequences encoding SEQ ID NO:2, 4, or 6 and for determining percent identity is provided in the specification and generally known in the art. See pages 9-13. Numerous delta-endotoxins were also well known in the art at the time the application was filed. See Crickmore *et al.* (1998) *Microbiol. Molec. Biol. Rev.* 62:807-813, which is incorporated by reference on page 2, lines 7-8 and was submitted with the response filed on June 2, 2006. The necessary molecular biology and mutagenesis techniques for preparing the variants and fragments of pesticidal sequences of the invention are routine. Moreover, methods for assessing the pesticidal activity of a polypeptide are readily available in the art and provided in the specification. See, for example, page 11, lines 15-19 and Examples 7 and 9-12.

In order to identify the pesticidal sequences encompassed by the present claims, one of skill in the art would only need to prepare variants and fragments of the nucleotide sequence of SEQ ID NO:1, 3, or 5, or a nucleotide sequence encoding SEQ ID NO:2, 4, or 6, having the specified characteristics recited in the claims (e.g., at least 95% identity) and then assay these polypeptides for pesticidal activity. Routine methods for preparing variants and fragments and testing the resulting polypeptides for pesticidal activity are routine in the art and described in the specification. Although some experimentation is required to practice the claimed invention, it is now customary in the art to generate a large number of sequences and to test them in a large-scale assay for a desired function, and, therefore, such experimentation is not undue, particularly in view of the routine nature of the required methods. Contrary to the Examiner's conclusions, in order to identify variants and fragments of the nucleotide sequence of SEQ ID NO:1, 3, or 5, or a nucleotide sequence encoding SEQ ID NO:2, 4, or 6 that could be used in the invention, a person skilled in the art would only need to utilize standard molecular biology and mutagenesis techniques and routine screening tests for pesticidal activity. Therefore, given the level of skill and knowledge in the art, the availability of standard methods and assays, and the significant

guidance provided in the specification, Applicants respectfully submit that the amount of experimentation required to identify delta-endotoxins and variants and fragments thereof having pesticidal activity and the structural features recited in the claims is routine, not undue.

In support of the argument that the invention is not enabled throughout the full scope of the claims, the Examiner cites *Genentech, Inc. v. Novo Nordisk, A/S*, 42 USPQ 2d 1001, 1005 (Fed. Cir. 1997) which, according to the Examiner, teaches that disclosure of a "mere germ of an idea does not constitute [an] enabling disclosure", and that "the specification, not the knowledge of one skilled in the art" must supply the enabling aspects of the invention (page 7 of the August 15, 2006 Office Action). Upon full review of this opinion, the Applicants contend that the *Genentech* decision actually supports the assertion that the invention is fully enabled.

The question before the Court in *Genentech* was whether the specification of U.S. Patent 5,424,199 would have enabled a skilled artisan to use cleavable fusion expression to make hGH without undue experimentation. The Court concluded that the specification does not describe in any detail whatsoever how to make hGH using cleavable fusion expression, and that no description of any specific cleavable conjugate protein appears. Contrarily, in the instant specification, specific nucleotide sequences encoding proteins with pesticidal activity, as well as fragments thereof, are provided (SEQ ID NO:1, 3, and 5), and sufficient guidance for preparing variants and fragments of SEQ ID NO:1, 3, or 5, or nucleotide sequences encoding SEQ ID NO:2, 4, or 6, and for determining percent identity is provided in the specification.

Further, although the Court does state that the "mere germ of an idea does not constitute an enabling disclosure", it immediately follows with the opinion that "[w]hile every aspect of a generic claim certainly need not have been carried out by an inventor, or exemplified in the specification, reasonable detail must be provided in order to enable members of the public to understand and carry out the invention." In citing *Hybritech Inc. v. Monoclonal Antibodies, Inc.* 802 F.2d 1367, 1385, 231 USPQ 81, 94 (Fed. Cir. 1986), the Court reiterates that "a specification need not disclose what is well known in the art" and characterizes "undue experimentation" as that in which "there is no disclosure of any specific starting material or of any of the conditions under which a process can be carried out".

As noted above, the instant specification clearly provides starting material (SEQ ID NO:1-6) as well as extensive description regarding the conditions under which amino acid substitutions can be carried out to generate a nucleotide sequence with at least 95% sequence identity to SEQ ID NO:1, 3, or 5, or a nucleotide sequence that encodes an amino acid sequence that is at least 95% identical to SEQ ID NO:2, 4, or 6. For instance, the specification describes examples of conserved residues that are not likely to tolerate substitution (see page 13), delineates conserved domains characteristic of delta-endotoxin proteins (see page 4), and highlights conserved residues in the sequences of the invention (see Figure 1 as originally filed). Further, routine methods for preparing variants and fragments and testing the resulting polypeptides for pesticidal activity are described in the specification.

The Examiner also cites *Amgen Inc. v. Chugai Pharmaceutical Co. Ltd.*, 18 USPQ 2d 1016 at 1027 in support of rejection of the claims for lack of enablement. Similar to the *Genentech* decision, the opinion provided by the Court in *Amgen* further supports the enablement of the present invention. As noted by the Examiner, the Court acknowledges that the disclosure in the Amgen patent (which provides a few EPO analog genes) "might well justify a generic claim encompassing these [EPO analog genes] and similar analogs". The Court ruled that the disclosure was inadequate support for Amgen's desire to claim *all* EPO gene analogs. There is no such claim in the instant application. Rather, the claims encompass specific nucleotide sequences (SEQ ID NO:1, 3, and 5), as well as nucleotide sequences with at least 95% sequence identity to SEQ ID NO:1, 3, or 5, or nucleotide sequences that encode an amino acid sequence that is at least 95% identical to SEQ ID NO:2, 4, or 6 (i.e., "similar analogs"). Therefore, the Applicants contend that the claims at issue in the *Amgen* decision are not related to the claims of the instant application, and that the opinion expressed by the Court in this decision actually *supports* the Applicants assertion that the claims are fully enabled.

The Examiner also maintains that mutation of sequences, even conservative substitutions, does not produce predictable results and, therefore, the specification is not enabling with respect to variants of the nucleotide sequence of SEQ ID NO:1, 3, or 5, or a nucleotide sequence encoding SEQ ID NO:2, 4, or 6. The Examiner continues to rely on Lazar *et al.* (1988) *Molecular and Cellular Biology* 8:1247-1252 and Hill *et al.* (1998) *Biochem. Biophys. Res.*

Comm. 244:573-577 in support of the general unpredictability of the art with respect to modification of nucleotide sequences. The Examiner asserts that the unpredictability is predicated on the fact that a "conservative" substitution reduced biological function of transforming growth factor alpha while "nonconservative" substitutions had no effect. However, regardless of the nature of the substitution (conservative vs. nonconservative), the alteration in the polypeptide was specifically designed to occur at amino acid positions that are highly conserved in the EGF-like family of polypeptides. Similarly, the modified residues described by Hill *et al.* were conserved among bacterial and plant ADP-glucose pyrophosphorylases. As noted previously, the first line of the abstract, "[t]wo *absolutely conserved* histidines and a third *highly conserved* histidine are noted in eleven bacterial and plant ADP-glucose pyrophosphorylases" (emphasis added). Again, one of skill in the art would not be surprised that modification of one of these highly conserved amino acids would lead to the loss of function described by the authors.

The Examiner stated that the surprise in Lazar *et al.* and Hill *et al.* was that these highly conserved amino acids in these well-characterized proteins did not behave as expected, and suggested that "the conventional wisdom about using conserved amino acids to guide the making of amino acid substitutions is wrong" (page 8 of the August 15, 2006 Office Action). The Applicants respectfully disagree with this unsubstantiated assertion by the Examiner. Conventional wisdom may, in fact, hinge on the vast preponderance of evidence that supports the Applicants position that the majority of conservative substitutions made in nonconserved regions have little or no effect on protein function.

For example, Jenkins *et al.* (1999) *FEBS Letters* 462:373-376 and Rajamohan *et al.* (1996) *J Biol. Chem.* 271(41):25220-25226 (submitted herewith as Appendices B and C, respectively), both demonstrate that non-conservative substitutions in non-conserved regions of delta-endotoxin proteins result in a loss of activity. While Rajamohan *et al.* do show that a conservative substitution in a non-conserved region can lead to a decrease (though not elimination) of activity, the non-conservative substitutions in these same regions have a much more significant impact on protein function.

Similarly, Lee *et al.* (2001) *FEBS Letters* 497:108-112 (Appendix D) show that several conservative substitutions in a non-conserved region (F276A and F280A) of Cry1Ac had no effect on toxicity, while many non-conservative substitutions in this non-conserved region of the protein eliminated toxicity of Cry1Ac toward *Manduca sexta* and *Lymantria dispar.* Schwartz *et al.* (1997) *Appl. Environ. Microbiol.* 63(10): 3978-3984 and Masson *et al.* (2002) *Appl. Environ. Microbiol.* 68(1):194-200 (Appendices E and F, respectively) show that both conservative and non-conservative substitutions in the conserved group 4 residues result in a decrease in toxicity of Cry1Aa and Cry1Ac proteins toward dipteran and lepidopteran pests, respectively.

Furthermore, the specification provides guidance regarding conservative modifications in *nonconserved* regions that are unlikely to disrupt biological activity (rather than conserved regions as is taught by both Lazar *et al.* and Hill *et al.*). See, for example, pages 12-14. Thus, by reference to a standard codon table, one of skill in the art could predict which modifications would not affect the biological activity of the encoded polypeptide. Also, the specification describes examples of conserved residues that are not likely to tolerate substitution (see page 13-14), delineates conserved domains characteristic of delta-endotoxin proteins (see page 4), and highlights conserved residues in the sequences of the invention (see Figure 1 as originally filed). One of skill in the art would understand that, in order to preserve function, amino acid substitutions of any nature should not be made in conserved regions of a protein. Therefore, the Examiner has failed to explain the relevance of Lazar *et al.* and Hill *et al.* to the present application since these two experiments targeted conserved regions of each protein under investigation.

The Examiner also urges that making amino acid substitutions in SEQ ID NO:2, 4, or 6 would need to be done randomly, that random substitutions have a likelihood of failure, and that the likelihood of failure amounts to undue experimentation. The Examiner relies on the teachings of Guo *et al.* (2004) *Proc. Natl. Acad. Sci. USA* 101:9205-9210 for the proposition that increasing the number of amino acid substitutions in a protein increases the probability that the protein will be functionally inactivated (i.e., "likelihood of failure"). As an initial matter, if one in fact wishes to make all 102 possible amino acid substitutions (as the Examiner contends is necessary to practice the full scope of the invention), the experimentation would be the same,

regardless of the likelihood of failure. Even Guo *et al.* suggest that the inactivation probability is only around 34%, which hardly constitutes a "likelihood of failure." Guo *et al.* further disclose that the 34% probability is based on data from a simple monomeric protein (which delta-endotoxins are not), and contends that the isolation of active mutants harboring many mutations from large random mutagenesis libraries is not surprising (page 9209, column 2 of Guo *et al.*).

Secondly, as discussed extensively herein, the specification provides sufficient guidance with respect to which amino acids are not likely to tolerate substitutions such that the making of amino acids with at least 95% sequence identity to SEQ ID NO:2, 4, or 6 *does not* require "random" substitution.

Further, detailed information about the structure of delta-endotoxins was also known in the art. See, for example, Li *et al.* (1991) *Nature* 353:815-821 (describing the crystal structure of the Cry3A protein), which is incorporated by reference on page 13 of the specification, and Morse *et al.* (2001) *Structure* 9:409-417, both of which were submitted with the June 2, 2006 response. Delta-endotoxins are extremely well-characterized and related to each other to various degrees by similarities in their amino acid sequences and tertiary structures. A combined consideration of the published structural analyses of delta-endotoxins and the reported functions associated with particular structures, motifs, and the like indicates that specific regions of the toxin are correlated with particular functions and discrete steps of the mode of action of the protein. Thus, a rational scheme for determining the regions of a delta-endotoxin that would tolerate modification is provided. Such a scheme eliminates the need to make random substitutions.

For example, based on the regions of delta-endotoxins that are conserved among protein family members, the skilled artisan could choose among possible modifications to produce polypeptides within the structural parameters set forth in the claims and then test these modified variants to determine if they retain pesticidal activity. In light of the guidance provided in the specification and the state of the art with respect to delta-endotoxins, a skilled artisan could readily conclude which amino acids are essential for structure and function and could envisage similar sequences that are 95% identical to the nucleotide sequence of SEQ ID NO:1, 3, or 5, or a nucleotide sequence encoding SEQ ID NO:2, 4, or 6, and that retain pesticidal activity. As

such, one of skill in the art could identify the pesticidal sequences encompassed by the present claims without undue experimentation.

The Examiner contends that the teachings of de Maagd *et al.* (1999) *Appl. Environ. Microbiol.* 65:4369-4374, Tounsi *et al.* (2003) *J. Appl. Microbiol.* 95:23-28 and Angsuthanasombat *et al.* (2001) *J. Biochem. Mol. Biol.* 34:402-407 support the assertion that amino acid substitutions in delta-endotoxin proteins are unpredictable. The Examiner further suggests that none of the specificity-altering mutations of these references falls within the conserved regions described in the specification. The Applicants respectfully disagree.

de Maagd *et al.* teach that the insertion of several groups of amino acids (blocks A-F) within Domain III of Cry1E with the corresponding amino acids of Cry1C will alter the specificity and/or toxicity of Cry1E. At least 3 of these blocks (blocks A, B, and F) map to the conserved regions described on page 4 of the instant specification (see Appendix A). Blocks B and D were described by de Maagd *et al.* as being important for the specificity of these toxins toward *S. exigua* and *M. sexta*. With the exception of blocks C and F, each of the blocks of substituted amino acids contained at least 2 (and up to 8) non-conservative substitutions. Thus, each of the specificity-altering substitutions either occurs in a conserved region, or involves a non-conservative substitution.

Similarly, Tounsi *et al.* discuss the single amino acid difference between Cry1Ia1 and Cry1Ia2 (which is a non-conservative substitution of aspartic acid for tyrosine at position 233) as being critical to insecticidal specificity of these two toxins. Finally, while Angsuthanasombat *et al.* teach a critical amino acid residue at position 136 where even a conservative substitution could lead to loss of pesticidal activity, the majority of the loss-of-function substitutions were non-conservative in nature. The Applicants do not presume that every conceivable conservative substitution in a nonconserved region will produce a protein with the recited activity, rather that the methods for making and testing substitutions within 95% sequence identity to SEQ ID NO:2, 4, or 6 is routine in the art, and the level of experimentation is not undue.

In establishing non-enablement, the burden rests initially with the Examiner to substantiate the unpredictability of the art and that, given the unpredictability, the specification does not provide sufficient information to guide those of skill to make and use the claimed

invention across the full scope of the claims. In view of the discussion above, the references cited by the Examiner fails to support the position that claims 1-11, 19, 22, and 23 are not enabled.

The Examiner further maintains that the specification does not enable the transformation of any plant with a nucleotide sequence with 95% identity to the nucleotide sequence of SEQ ID NO:1, 3, or 5, or a nucleotide sequence encoding SEQ ID NO:2, 4, or 6 because undue trial and error experimentation would be required to screen for nucleotide sequences encompassed by the claims and plants transformed therewith to identify those plants with pesticidal activity. As discussed above, the amount of experimentation required to identify a nucleotide sequence that has 95% sequence identity to SEQ ID NO:1, 3, or 5, or to a nucleotide sequence encoding SEQ ID NO:2, 4, or 6 is not undue. Given the guidance provided in the specification and the knowledge in the art, the claims directed to transformation of a plant with a delta-endotoxin sequence, or variant or fragment thereof, are fully enabled.

In light of the above arguments, the level of skill and knowledge in the art, and the guidance provided in the specification, Applicants respectfully submit that the specification is enabling for the full scope of claims 1-11, 19, 22 and 23. Thus, the rejection of the claims under 35 U.S.C. § 112, first paragraph, for lack of enablement should be withdrawn.

Written Description

Claims 1-11, 19, 22 and 23 were further rejected under 35 U.S.C. § 112, first paragraph, as failing to satisfy the written description requirement. The rejection is respectfully traversed.

The Examiner asserts that the disclosure is insufficient to support claims that are drawn to a genus of nucleic acids having 95% sequence identity to SEQ ID NO:1, 3, or 5, or nucleic acids encoding polypeptides having 95% identity to SEQ ID NO:2, 4, or 6. In order to satisfy the written description requirement of 35 U.S.C. § 112, the application must reasonably convey to one skilled in the art that the applicant was in possession of the claimed subject matter at the time the application was filed. *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563, 19 U.S.P.Q.2d (BNA) 1111, 1117 (Fed. Cir. 1991). Every species encompassed by the claimed invention, however, need not be disclosed in the specification to satisfy the written description requirement

of 35 U.S.C. § 112, first paragraph. *Utter v. Hiraga*, 845 F.2d 993, 6 USPQ2d 1709 (Fed. Cir. 1988). The Federal Circuit has made it clear that sufficient written description requires simply the knowledge and level of skill in the art to permit one of skill to immediately envision the product claimed from the disclosure. *Purdue Pharm L.P. v. Faulding In.*, 230 F.3d 1320 1323, 596 USPQ2d 1481, 1483 (Fed. Cir. 2000) ("One skilled in the art must immediately discern the limitations at issue in the claims.").

Moreover, the "Guidelines for Examination of Patent Applications Under 35 U.S.C. §112, ¶ 1, 'Written Description' Requirement" state that a genus may be described by "sufficient description of a representative number of species . . . or by disclosure of relevant, identifying characteristics , *i.e.* structure or other physical and/or chemical properties." *Id.* at 1106. This is in accordance with the standard for written description set forth in *Regents of the University of California v. Eli Lilly & Co*, 119 F.3d 1559 (Fed. Cir. 1997), where the court held that "[a] written description of an invention involving a chemical genus, like a description of a chemical species, 'requires a precise definition, such as by structure, formula, or chemical name' of the claimed subject matter sufficient to distinguish it from other materials." 119 F.3d at 1568, citing *Fiers v. Revel* 984 F.2d 1164 (Fed. Cir. 1993). In *Enzo Biochem, Inc. v. Gen-Probe, Inc.*, 323 F.2d 926 (Fed. Cir. 2002), the Federal Circuit adopted the PTO standard for written description, stating:

> [U]nder the Guidelines, the written description requirement would be met . . . if the functional characteristics of [a genus of polypeptides] were coupled with a disclosed correlation between that function and a structure that is sufficiently known or disclosed. We are persuaded by the Guidelines on this point and adopt the PTO's applicable standard for determining compliance with the written description requirement."

The claims of the present application meet the requirements for written description set forth by the Federal Circuit. The claims recite that the nucleic acid have 95% sequence identity to the nucleotide sequence of SEQ ID NO:1, 3, or 5, or to a nucleotide sequence encoding SEQ ID NO:2, 4, or 6. Methods for determining percent identity between any two sequences are known in the art and are provided in the specification. See pages 9-13. As discussed above, nucleotide sequences for full-length AXMI-009 (SEQ ID NO:1), as well as variants and

fragments (e.g., SEQ ID NO:3 and 5) are disclosed in the specification. Numerous delta-endotoxin sequences were also generally known in the art at the time the application was filed. Moreover, detailed information regarding the structure of delta-endotoxins and the reported functions associated with particular structures, regions, and motifs was also available in the prior art as well as discussed in detail on page 2, lines 21-29, Figure legend 1, and on page 12.

At the time of filing, it was known that delta-endotoxins generally comprise three domains, a seven-helix bundle that is involved in pore formation, a three-sheet domain that has been implicated in receptor recognition, and a beta-sandwich motif. See Li *et al.* (1991) *Nature* 305:815-821. Thus, the recitation of polypeptides having a particular percent identity to a delta-endotoxin provides very specific and defined structural parameters of the sequences that can be used in the invention. These structural limitations are sufficient to distinguish the nucleotide and amino acid sequences of the invention from other nucleic acids and polypeptides and thus sufficiently define the genus of sequences useful in the practice of the present invention.

The Examiner maintains that the specification describes no relevant characteristics or motifs for the claimed nucleic acids other than identity to SEQ ID NO:1, 3, or 5, and that the level of skill and knowledge in the art at the time of filing is such that no other proteins within the scope of the claims were known. Applicants acknowledge that no other proteins within the scope of the claims were known; hence, the novelty of the invention. However, Applicants respectfully disagree with the assertion that no relevant characteristics or motifs were disclosed. As discussed above, domains associated with specific functions were known (Li *et al.*, *supra*), and conserved regions within each of these functional domains are described in the specification. Although the Examiner dismisses the relevance of these teachings since they describe a cry3Aa protein (pages 8- 9 of the August 15, 2006 Office Action), Li *et al.* state that the overall structure of this delta-endotoxin represents the general fold of the family of active delta-endotoxin proteins (see the abstract of Li *et al.*), and that the core of the cry3Aa molecule is built from the five sequence blocks that are highly conserved throughout the delta-endotoxin family (column 2, page 817 of Li *et al.*). These highly conserved sequence domains have been described in the instant specification as they relate to the delta-endotoxin of the invention.

The Examiner is also reminded that the description of a representative number of species does not require the description to be of such specificity that it would provide individual support for each species that the genus embraces. 66 Fed. Reg. 1099, 1106 (2000). Satisfactory disclosure of a "representative number" depends on whether one of skill in the art would recognize that the applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus in view of the species disclosed. 66 Fed. Reg. 1099, 1106 (2000). Here, Applicants have provided nucleotide and amino acid sequences for exemplary pesticidal sequences and variants and fragments thereof encompassed by the claims. Moreover, numerous delta-endotoxin sequences were known and readily available in the art. Therefore, Applicants submit that in view of the present disclosure and the knowledge and level of skill in the art the skilled artisan would envision the claimed invention.

The description of a claimed genus can be by structure, formula, chemical name, or physical properties. *See Ex parte Maizel*, 27 USPQ2d 1662, 1669 (B.P.A.I. 1992), *citing Amgen v. Chugai*, 927 F.2d 1200, 1206 (Fed. Cir. 1991). A genus of polypeptides may therefore be described by means of a recitation of a representative number of amino acid sequences that fall within the scope of the genus, *or* by means of a recitation of structural features common to the genus, which features constitute a substantial portion of the genus. *See Regents of the University of California v. Eli Lilly & Co.*, 119 F.3d 1559, 1569 (Fed. Cir. 1997); *see also* Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, first paragraph, "Written Description" Requirement, 66 Fed. Reg. 1099, 1106 (2000). The recitation of a predictable structure (i.e., an amino acid sequence having a specified percent identity or number of contiguous amino acid residues of a particular sequence) is sufficient to satisfy the written description requirement. Thus, the application provides the structural features that characterize sequences having at least 95% sequence identity to SEQ ID NO:1, 3, or 5, or to a nucleotide sequence encoding SEQ ID NO:2, 4, or 6 that retain pesticidal activity.

An Applicant may also rely upon functional characteristics in the description, provided there is a correlation between the function and structure of the sequences recited in the claims. *Id.*, citing *Lilly* at 1568. The present claims further recite functional characteristics that distinguish the sequences of the claimed genus. Specifically, the claims recite that the sequences

having at least 95% sequence identity to SEQ ID NO:1, 3, or 5, or to a nucleotide sequence encoding SEQ ID NO:2, 4, or 6 encode proteins which have pesticidal activity. The specification and the art provide standard assays that may be used to measure pesticidal activity. See, for example, page 8, lines 27-31. Furthermore, as noted above, Applicants have disclosed fragment sequences that retain pesticidal activity (e.g., SEQ ID NO:3 and 5, which encode fragments of SEQ ID NO:2). Accordingly, both the structural and functional properties that characterize the genus of sequences that can be used to practice the invention are specifically recited in the claims. The sequences that fall within the scope of the claims can readily be identified by the methods set forth in the specification.

In summary, the specification provides an adequate written description of the claimed invention. In particular, the specification provides: nucleotide and amino acid sequences for pesticidal toxins, and variants and fragments thereof, that fall within the scope of the claims; guidance regarding sequence alterations that do not disrupt pesticidal activity of a toxin; guidance for determining percent identity; and methods for assaying the pesticidal activity of proteins. In view of the above remarks and claim amendments, Applicants submit that the relevant identifying structural and functional properties of the genus of sequences of the present invention would be clearly recognized by one of skill in the art. Consequently, Applicants were in possession of the invention at the time the application was filed, and the rejection of the claims under 35 U.S.C. § 112, first paragraph, for lack of written description should be withdrawn.

The Rejection of the Claims Under 35 U.S.C. § 112, Second Paragraph Should Be Withdrawn

The rejection of claim 11 under 35 U.S.C. § 112, second paragraph has been maintained as being indefinite for failing to particularly point out and distinctly claim the subject matter that Applicant regards as the invention.

Claim 11 has been amended to recite "a transgenic seed comprising the nucleic acid molecule of claim 1. Therefore, as amended, claim 11 now describes a transgenic seed that comprises the nucleic acid of claim 1. Accordingly, the rejection of claim 11 under 35 U.S.C. § 112, second paragraph should be withdrawn.

It is not believed that extensions of time or fees for net addition of claims are required, beyond those that may otherwise be provided for in documents accompanying this paper. However, in the event that additional extensions of time are necessary to allow consideration of this paper, such extensions are hereby petitioned under 37 CFR § 1.136(a), and any fee required therefore (including fees for net addition of claims) is hereby authorized to be charged to Deposit Account No. 16-0605.

Respectfully submitted,

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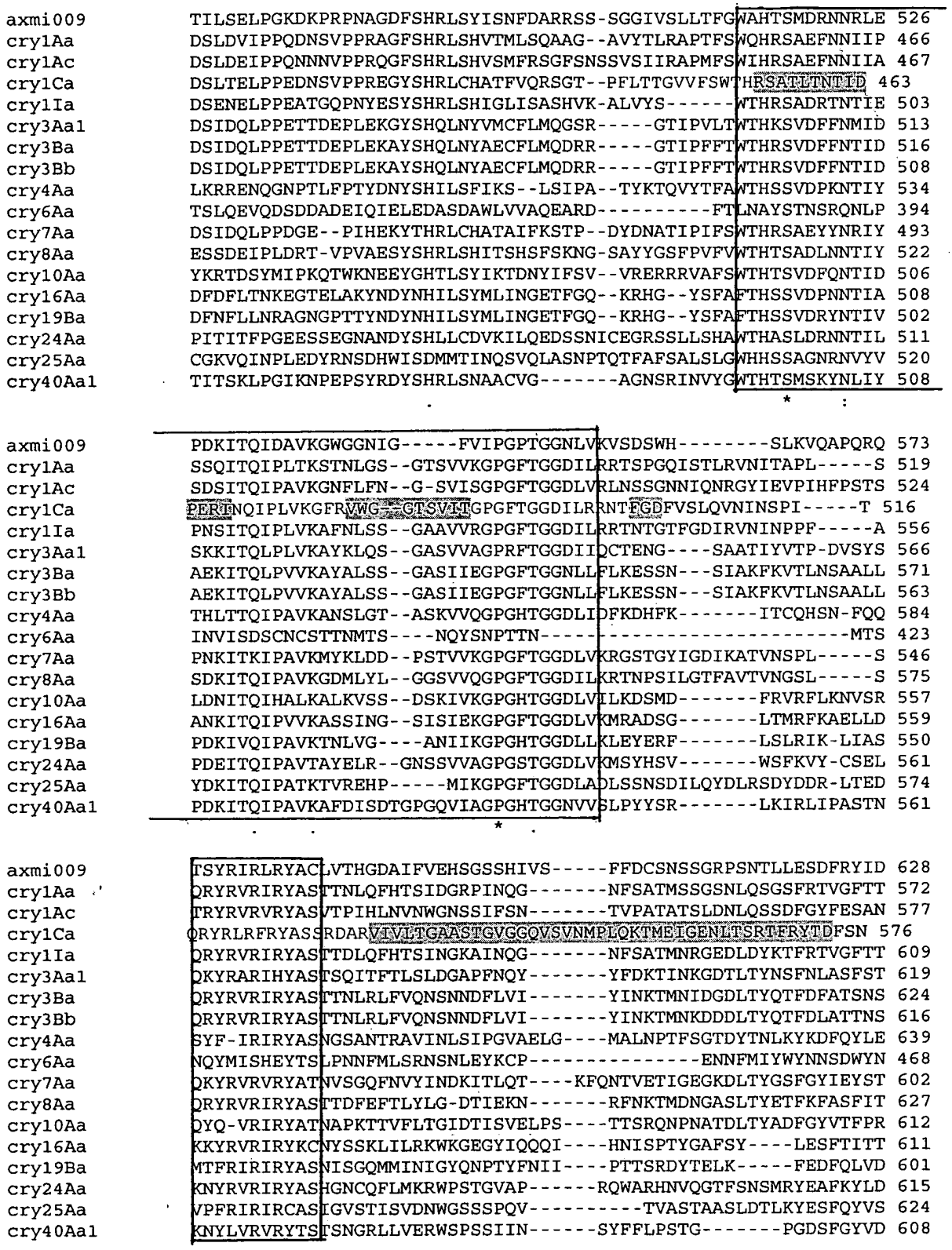
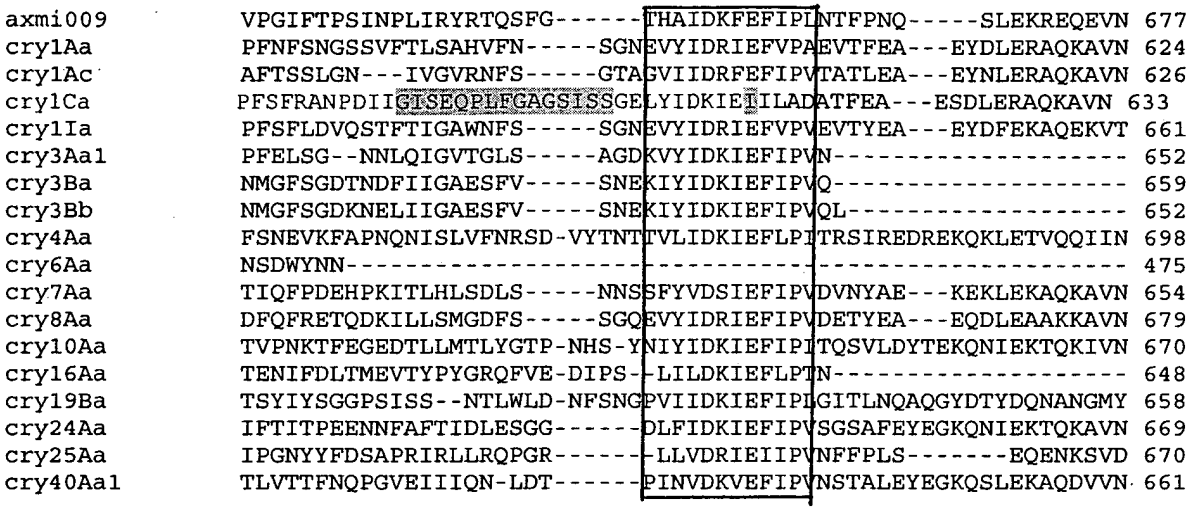
Nora C. Martinez



HIGHLIGHTED SEQUENCES = Blocks A-F (de Maagd *et al.* (1999) *Appl. Environ. Microbiol.* 65:4369-4374)

BLOCKED SEQUENCES = Conserved domains 3-5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cry1Ac AFTSSLGN---IVGVRNFS-----GTAGVIIDRFEFIPVTATLEA---EYNLERAQKAVN 626
cry1Ca PFSFRANPDIIGTSEQPLFGAGSISSGELYIDKIEIILADATFEA---ESDLERAQKAVN 633
cry1Ia PFSFLDVQSTFTIGAWNFS-----SGNEVYIDRIEFVPVEVTYEA---EYDFEKAQEKVT 661
cry3Aa1 PFELSG--NNLQIGVTGLS-----AGDKVYIDKIEFIPVN---------- 652
cry3Ba NMGFSGDTNDFIIGAESFV-----SNEKIYIDKIEFIPVQ---------- 659
cry3Bb NMGFSGDKNELIIGAESFV-----SNEKIYIDKIEFIPVQL--------- 652
cry4Aa FSNEVKFAPNQNISLVFNRSD-VYTNTTVLIDKIEFLPITRSIREDREKQKLETVQQIIN 698
cry6Aa NSDWYNN---------- 475
cry7Aa TIQFPDEHPKITLHLSDLS-----NNSSFYVDSIEFIPVDVNYAE---KEKLEKAQKAVN 654
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cry10Aa TVPNKTFEGEDTLLMTLYGTP-NHS-YNIYIDKIEFIPITQSVLDYTEKQNIEKTQKIVN 670
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Binding of *Bacillus thuringiensis* Cry1Ac toxin to *Manduca sexta* aminopeptidase-N receptor is not directly related to toxicity

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Received 20 August 1999; received in revised form 3 November 1999

Edited by Marc Van Montagu

Abstract ***Bacillus thuringiensis*** **Cry1Ac δ-endotoxin specifically binds a 115-kDa aminopeptidase-N purified from *Manduca sexta* midgut. Cry1Ac domain III mutations were constructed around a putative sugar-binding pocket and binding to purified aminopeptidase-N and brush border membrane vesicles (BBMV) was compared to toxicity. Q509A, R511A, Y513A, and 509–511 (QNR-AAA) eliminated aminopeptidase-N binding and reduced binding to BBMV. However, toxicity decreased no more than two-fold, indicating activity is not directly correlated with aminopeptidase-N binding. Analysis of toxin binding to aminopeptidase-N in *M. sexta* is therefore insufficient for predicting toxicity. Mutants retained binding, however, to another BBMV site, suggesting alternative receptors may compensate in vivo.**

Key words: Aminopeptidase-N; Brush border membrane vesicle; Surface plasmon resonance; *N*-Acetylgalactosamine; *Bacillus thuringiensis*

1. Introduction

Bacillus thuringiensis (Bt) produces insecticidal crystal proteins during its sporulation process. The crystal inclusions are solubilized in the insect midgut to produce protoxin which is further digested to an active toxin fragment by midgut proteases. The activated toxin binds to specific receptors on the surface of the midgut epithelial cell membrane. Membrane-bound toxin is believed to be inserted into the lipid membrane and form pores or ion channels, leading to insect death. The receptor-binding step is considered to be critical to its mode of action and in resistance development.

Receptor-binding properties have been examined with brush border membrane vesicles (BBMV) prepared from the insect larval midguts and labeled Bt toxins [1]. Recently, surface plasmon resonance (SPR) techniques have been employed as well to study interactions between toxins and purified receptors [2–6]. Aminopeptidase-N (APN) was first identified [7] and purified [8] as a Cry1Ac receptor from *Manduca sexta* BBMV. APN from other insects were also identified as Cry1Ac receptors [9–11]. Cry1Ac binding to APN is inhibited specifically by *N*-acetylgalactosamine (GalNAc) [2,3,12], providing evidence that a carbohydrate moiety on APN is involved in toxin binding. A 210-kDa cadherin-like Cry1A-binding protein from *M. sexta* has also been reported [13,14].

The X-ray crystal structure of Cry1Aa reveals a three-domain composition [15]. Domain I, composed of seven α-helices, is important for membrane insertion and pore formation. Domain II, composed of antiparallel β-sheets, is involved in binding to BBMV and to purified receptors. Domain III, composed of a β-sheet sandwich, is also involved in receptor binding and ion-channel conductivity. Domain III shares the typical jelly roll topology found in other sugar-binding proteins that often lack primary sequence homology. Similar folds can be seen in sugar-binding bacterial toxins, viral proteins, glycosidases, and plant, animal, and fungal lectins. Lectins, however, have more than one sugar-binding site, and thus, can agglutinate cells [16]. Due to its abundance in intestinal tracts [17], many pathogenic lectins appear to have evolved to target GalNAc.

Previously, we have constructed alanine substitution mutations (residues 502–523) in the β-sheet in domain III of Cry1Ac toxin, and their biological activities and BBMV binding to several insects, including *M. sexta*, *Lymantria dispar*, *Heliothis virescens* were examined [18,19]. Mutations at the residues Q509, R511, and Y513 affected toxicity and BBMV binding to these insects. In the present paper, we have further examined the binding properties of these mutant toxins to *M. sexta* APN using SPR to evaluate the relationship between toxicity and purified receptor binding.

2. Materials and methods

2.1. Mutant toxin construction, expression, and purification

A putative 3D model of Cry1Ac was constructed by homology modeling from Cry1Aa [15] using SWISS-MODEL [20] and visualized using SWISS-PdbViewer v.3.1 with Q3D rendering. Site-directed mutagenesis of *cry1Ac1* [21] was carried out by the Kunkel method [22] and transformed into *Escherichia coli* MV1190 for expression. Active 65-kDa Cry1Ac toxins were produced as described previously [23]. Active proteins were further purified by size-exclusion chromatography on a Superdex 200 column (Amersham Pharmacia Biotech). Monomeric fractions were collected, and MW verified by dynamic light scattering (DynaPro-801). The monomeric form of the toxin was not found to multimerize in storage once column-purified from aggregate toxin forms. Mutants were analyzed by 10% SDS-PAGE and shown to be equal in size with wild type after trypsinization and size-exclusion purification.

2.2. BBMV preparation and binding studies

M. sexta BBMV were prepared by the magnesium precipitation method [24]. Iodination of toxins, BBMV competition binding, and ligand blotting were conducted as described previously [23,25].

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PII: S0014-5793(99)01559-8

2.3. SPR analysis of binding to purified M. sexta APN

M. sexta APN was purified and tested for Cry1Ac binding ability as described in [6]. SPR technique was carried out using a BIAcore 2000 (Biacore AB, Uppsala, Sweden) with a CM5 sensor chip. All toxins were dialyzed into HBS (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA pH 7.4). Amine coupling of the soluble, 115-kDa Cry1Ac-binding APN was achieved in ammonium acetate, pH 4.2. Five randomized toxin concentrations (10–1000 nM) were 'kinjected' (low dispersion injection for kinetic measurements) at 30 μl/min. The experiment was repeated on three different APN-containing flow cells with two independent APN preparations. APN was regenerated by two 15–30-μl pulses of 10 mM NaOH, pH 11.0. Wild-type Cry1Ac binding was checked before and after all mutants to ensure the integrity of APN. Fittings were done globally using BIAevaluation 3.0. All models available with the software were tested, and the heterogeneity model (T+R1⇒TR1, T+R2⇒TR2) was chosen as the best fit ($\chi^2 < 1$). Initial global values for k_{on} and k_{off} were obtained from local fittings. Standard error is < 10% of the reported value for all apparent rate constants.

3. Results

3.1. Cry1Ac domain III mutants and biological activities to M. sexta

Alanine substitutions were made in *cry1Ac1* at Q509, N510, R511, Y513, and at residues ^{509}QNR^{511}. When expressed, all mutants formed toxins that were stable after trypsin activation and analyzed by 10% SDS-PAGE (not shown). CD spectra of the mutant toxins were comparable to that of wild-type Cry1Ac, suggesting that the mutations do not alter structural integrity [19]. In sequence alignments with *cry1Aa* and *cry1Ab*, these residues are found in a non-conserved loop region extending from β-15 and continuing into β-16. A 3D structure of Cry1Ac was homology-modeled from Cry1Aa. Where a relatively flat surface is displayed on Cry1Aa, a cavity is predicted to form on the surface of Cry1Ac domain III. The mutants presented in this study line the bottom of the cavity (Fig. 1). Bioassays revealed that the mutant toxins are about two-fold less toxic than the wild-type Cry1Ac toxin (LC_{50} = 4.7 ng/cm^2). The range of LC_{50} values for the mutant toxins is 7.5–9.8 ng/cm^2. Detailed bioassay data are presented elsewhere [19].

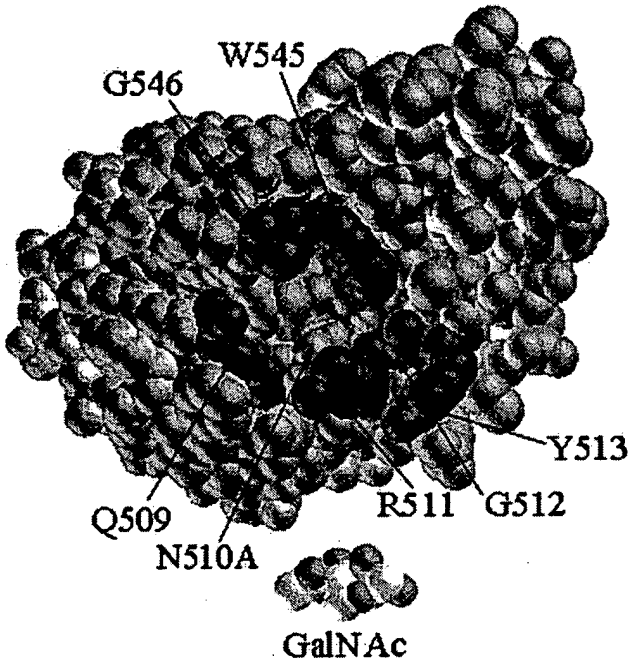

Fig. 1. Molecular model of Cry1Ac domain III GalNAc-binding pocket. Residues lining the cavity mouth are in black. GalNAc structure is shown for size comparison.

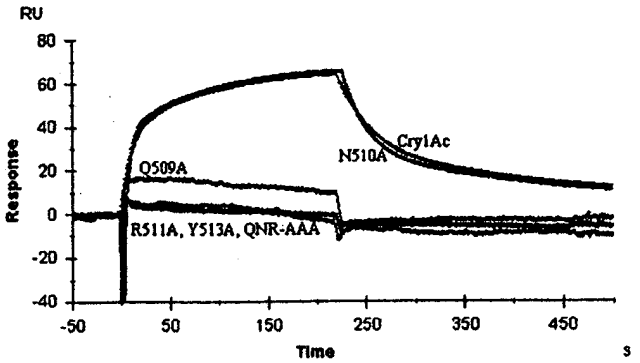

Fig. 2. Sensorgram overlay comparing wild-type Cry1Ac and mutant toxin binding (100 nM) to purified *M. sexta* APN.

3.2. Real-time binding to purified APN

A BIAcore 2000 enabled the use of the SPR technique to monitor changes in mass on a surface of immobilized receptor as toxin is injected. Apparent rate constants were obtained by global analysis using BIAevaluation 3.0. Monomeric Cry1Ac bound *M. sexta* APN heterogeneously (Fig. 2) (T+R1⇒TR1, T+R2⇒TR2), confirming previously published results [2]. Wild-type apparent rate constants obtained were $k_{on1} = 3e^5$ M^{-1} s^{-1}, $k_{off1} = 3e^{-2}$ s^{-1} and $k_{on2} = 4.4e^4$ M^{-1} s^{-1}, $k_{off2} = 3.4e^{-3}$ s^{-1}, yielding K_D affinities of 141 nM and 77 nM, respectively. N510A bound APN with wild-type affinity (apparent rate constants were within standard error range of wild-type). No binding to APN was seen for Q509, R511, Y513, or QNR-AAA at any concentration. Therefore, no rate constants were measured for these mutant toxins.

3.3. BBMV receptor-toxin overlay

M. sexta BBMV proteins were separated by 6% SDS-

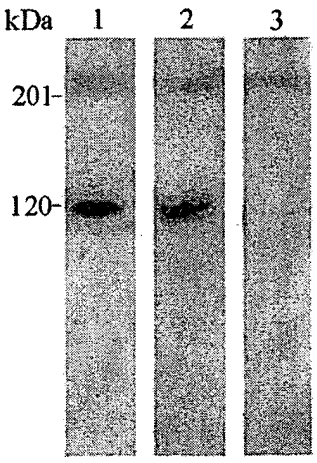

Fig. 3. Binding of ^{125}I-labeled Cry1Ac (lane 1), N510A (lane 2), and QNR-AAA toxin (lane 3) to *M. sexta* BBMV proteins. BBMV proteins (20 μg) were separated by 6% SDS-PAGE, transferred to PVDF, and probed with labeled toxins.

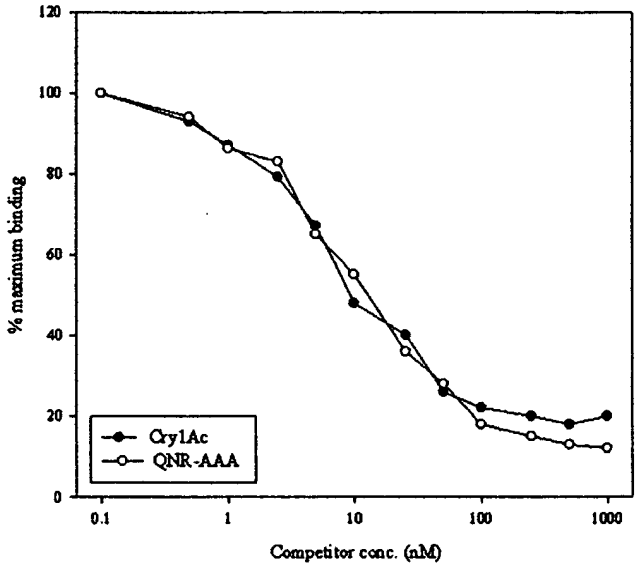

Fig. 4. Binding of Cry1Ac and domain III mutant toxins to *M. sexta* BBMV. ^{125}I-QNR-AAA (2 nM) was incubated with 10 μg BBMV in the presence of increasing concentrations of unlabeled Cry1Ac (●) or unlabeled QNR-AAA (○).

PAGE, transferred to PVDF membrane, and probed with ^{125}I-labeled toxins. Cry1Ac bound to a band slightly above 200 kDa, as well as a 120-kDa band, the molecular size of APN (Fig. 3). These results are in agreement with earlier findings for Cry1Ac [26]. N510A showed similar binding to wild-type. Conversely, labeled QNR-AAA showed no visible binding within the APN size range, but did retain binding to the higher band.

3.4. Binding to BBMV

In previous BBMV competition assays, we have shown that QNR-AAA and individual mutant toxins Q509A, R511A, and Y513A did not compete as well with labeled Cry1Ac for binding to BBMV compared to wild-type self-competition [19]. The affinity of Cry1Ac and QNR-AAA for *M. sexta* BBMV receptor sites was 3.9 (±1.2) and 38.2 (±5.3) nM respectively, reflecting a 10-fold difference. To further investigate the nature of QNR-AAA's reduced affinity, a reciprocal competition binding assay was conducted. Labeled QNR-AAA was competed with cold QNR-AAA or cold wild-type (Fig. 4). Cold Cry1Ac competes with labeled QNR-AAA with the same affinity that cold QNR-AAA does. The combined results of the reciprocal binding studies do not support the hypothesis that the QNR-AAA mutant toxin has reduced affinity for only APN. Rather, they suggest that Cry1Ac binds to multiple receptor sites, and that the QNR-AAA mutant toxin loses binding to APN sites while retaining binding to others.

4. Discussion

In the present study, a functional role for domain III of Cry1Ac was investigated by a series of mutations in a β-sheet region particular to Cry1Ac. Our results show considerable differences between binding affinities of wild-type and mutant toxin to *M. sexta* APN compared to differences in toxicity. The requirement of domain III residues for binding to *M. sexta* APN correlates with the findings in studies using domain-switched mutants [6,27,28] and ligand overlay blots using *M. sexta* BBMV [29]. Additionally, the loss of binding by domain III mutants to *Lymantria dispar* and *Heliothis virescens* BBMV [19] suggests these amino acids have a common role in binding to insect APNs. This idea is supported by studies that showed GalNAc inhibits Cry1Ac binding to both *L. dispar* and *H. virescens* APNs using the SPR technique [3,4]

A molecular model of Cry1Ac predicted these mutations to be located around a unique cavity that is not present on Cry1Aa. Residues W545 and G546 complete the upper portion of the cavity mouth (Fig. 1). N510 appears inside, and not surrounding, the cavity. The lip surrounding this indentation presents residues in Cry1Ac entirely non-conserved with Cry1Aa. Furthermore, the Cry1Aa amino acids 'filling in' the cavity from β-sheet 22 (S581, V582, F583) are missing in Cry1Ac, allowing G584 and N585 to line the pocket unobtrusively. GalNAc is known to inhibit Cry1Ac binding to purified APN, but not Cry1Aa [2]. This model provides evidence of structural differences between Cry1Ac and other Cry1A toxins that may account for the sugar-binding specificity of Cry1Ac.

Our experiments provide evidence that demonstrates Cry1Ac has multiple receptor specificity, which is lost when domain III mutations are constructed around the putative cavity. SPR studies and BBMV ligand blots showed QNR-AAA lost binding to APN from *M. sexta* BBMV, suggesting the unique cavity in Cry1Ac domain III is necessary for APN binding. However, QNR-AAA retained binding to another Cry1Ac-binding band that is also bound by wild-type. The presence of at least one other Cry1Ac-binding component in BBMV indicates that APN does not act as a lone receptor. Additionally, despite QNR-AAA's 10-fold loss of affinity for *M. sexta* BBMV in competition assays, it remains an equal competitor as wild-type for its binding to other receptor sites (Fig. 4). Therefore, the Cry1Ac mechanism of binding to APN (by domain III-sugar contact) may be non-essential for binding to other sites. Evidence of GalNAc-independent binding mechanisms has been observed in BBMV prepared from the anterior subregion of *M. sexta* midgut [30]. Furthermore, a report using various ligand binding protocols [26] found that a 210-kDa cadherin-like glycoprotein, BT-R1, from *M. sexta* binds Cry1A proteins. These data were strongly supported by the fact that cloned BT-R1 expressed in insect cells still bound Cry1A toxins [31]. A similar cadherin-like glycoprotein, BtR175, has been cloned from *Bombyx mori* that binds Cry1Aa [32] in a GalNAc-independent manner. Antibodies to BtR175 block insecticidal action in vivo [33].

Given the binding and toxicity contradiction found in this study, one possible explanation of our data is that while receptor recognition did not occur in in vitro binding studies, over the time course of a bioassay (5 days), only minor reductions in insecticidal activity occur. Alternatively, the first instar larvae used in bioassays and fifth instar larvae used for BBMV preparations may differ in a way that causes loss of binding at a later stage but not an earlier one. However, mutations in the domain II of several Cry toxins have been shown to reduce both toxicity and binding to BBMV in numerous publications. A second model to explain the contradiction is that Cry1Ac binding to another receptor is more functionally critical than binding to APN receptor. However, if APN acts as a binding protein without allowing the pore-

formation step, it should be considered a sequestering protein. In this model, mutants that lose binding to APN should be free to bind functional sites, thus augmenting toxicity. This is not the result of our bioassays. About two-fold less activity was observed for these mutant toxins. For APN to be non-critical in the gut environment it would have to be present in significantly lower ratios than other receptors, making loss of binding irrelevant. Neither BBMV ligand blots (Fig. 3) nor gut enzyme studies suggest a paucity of APN in insect guts. Furthermore, APNs from *M. sexta* and *H. virescens* have been reconstituted into membranes and caused toxin-induced pores to form [34]. In a final possible scenario, APN serves as a functional receptor among others. Although the binding to APN was completely abolished, binding to other receptors could be increased, which, in turn, maintain the toxicity over the time course of a bioassay. In this model, a mutant with enhanced affinity for APN might still enhance toxicity.

We note a recent report published while this paper was in preparation. The authors found similar Cry1Ac domain III mutations had no effect on toxicity to *M. sexta* [29]. The mutant toxins had reduced affinity for *M. sexta* BBMV as well as ability to induce BBMV permeability. Also, the mutants lost binding to the APN component of BBMV, as does Cry1Ac when competed with GalNAc. Here, we further advance a model for Cry1Ac receptor binding in this study by demonstrating a total loss of mutant toxin binding to purified APN using real-time kinetics. We see binding to other receptor sites by ligand blotting and reciprocal competition binding assays, which might account for the small changes in toxicity. Finally, we show a structural difference between Cry1Ac and Cry1Aa, which explains why Cry1Ac has sugar-binding specificity.

Mutations where loss of binding to purified APN is well correlated with loss of toxicity have been observed in Cry1Ac and Cry1Ab when domain II loop residues were altered (J. Jenkins and D. Dean, unpublished). A comprehensive binding model of domain III and domain II is under investigation to better understand the relationship between receptor binding and insecticidal activity.

Acknowledgements: This research was funded by a grant from the National Institutes of Health, Allergies and Infectious Diseases, R01 AI29092-08. *M. sexta* eggs were supplied by D.L. Dahlman (Department of Entomology, University of Kentucky, Lexington, KY). Thanks to Christopher Whalen for use of the BIAcore 2000 instrument.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 41, Issue of October 11, pp. 25220–25226, 1996
Printed in U.S.A.

Mutations at Domain II, Loop 3, of *Bacillus thuringiensis* CryIAa and CryIAb δ-Endotoxins Suggest Loop 3 Is Involved in Initial Binding to Lepidopteran Midguts*

(Received for publication, April 1, 1996, and in revised form, June 6, 1996)

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Alanine substitutions of loop 3 residues, ^{438}SGFSNS^{443}, of CryIAb toxin were constructed to study the functional role of these residues in receptor binding and toxicity to *Manduca sexta* and *Heliothis virescens*. Experiments with trypsin and insect gut juice enzyme digestions of mutant toxins showed that these mutations did not produce any gross structural changes to the toxin molecule. Bioassay data showed that mutant G439A (alanine substitution of residue Gly^{439}) and F440A significantly reduced toxicity toward *M. sexta* and *H. virescens*. In contrast, mutants S438A, S441A, N442A, and S443A were similar or only marginally less toxic (2–3 times) to the insects compared to the wild-type toxin. Binding studies with brush border membrane vesicles prepared from *M. sexta* and *H. virescens* midgut membranes revealed that the loss of toxicity of mutants G439A and F440A was attributable to substantially reduced initial binding. Consistent with the initial binding, mutants G349A and F440A showed 3.5 times less binding to *M. sexta* and *H. virescens* brush border membrane vesicles, although the off-rate of bound toxins was not affected. The role of hydrophobic residue, Phe^{440}, is distinctly different from our previous observation that alanine substitution of Phe^{371} at loop 2 of CryIAb did not affect initial binding but reduced irreversible association of the toxin to the receptor or membrane toward *M. sexta* (Rajamohan, F., Alcantara, E., Lee, M. K., Chen, X. J., and Dean, D. H. (1995) *J. Bacteriol.* 177, 2276–2282). Likewise, deletion of relatively hydrophobic CryIAa loop 3 residues, ^{440}AAGA^{443} (D3a), resulted in reduced toxicity to *Bombyx mori* (>62 times less) and *M. sexta* (28 times less). The loss of toxicity was correlated with reduced initial binding to midgut vesicles prepared from these insects. However, alanine substitution of residues ^{437}LSQ^{439} (A3a), contiguous to loop 3, altered neither toxicity nor receptor binding toward *B. mori* or *M. sexta*. These results suggest that the loop 3 residues of CryIAb and CryIAa toxins establish hydrophobic interactions with the receptor molecule, and mutations at these hydrophobic residues affect initial binding.

The insecticidal crystal proteins (ICPs or δ-endotoxins) produced by *Bacillus thuringiensis* are of great scientific interest because of their potency and specificity against a wide range of agronomically important insect pests and vectors of human diseases (1). The bacteria express the protein during the late growth phase as a protoxin (120–140 kDa for CryI types), which accumulates in the cell as crystals of various shapes (2). Upon ingestion of the crystals, the protoxin is solubilized and activated into a 60–65-kDa protease-resistant toxin by the proteolytic enzymes present in the larval midgut. The activated toxin binds to specific receptors (toxin-binding proteins) located on the midgut brush border membrane of the columnar cells (3, 4). Binding of toxin to the receptor generates ion channels across the midgut apical membrane, leading to death of the cells (5–7) and finally of the larvae.

The x-ray crystal structure of a lepidopteran active δ-endotoxin, CryIAa, has been recently determined by Grochulski *et al.* (8). This structure supports the three domain structure of CryIIIA, a coleopteran active toxin, determined by Li *et al.* (9). In summary, domain I is composed of seven α-helixes, which may be involved in the membrane spanning activity of the toxin. Mutations in domain I of CryI type toxins can inhibit toxicity and channel forming activity (10, 11). Domain II has three antiparallel β-sheets, connected to each other with surface-exposed loops of different lengths, oriented in parallel with the helical bundle of domain I. These loops are attractive candidates for a role in receptor recognition and binding. Domain III, a bundle of β-sheets, has been reported to be involved in ion-channel activity (12), receptor binding (13), and structural stability (14). CryIAb toxin is believed to have a similar structure as CryIAa, since it shares about 89% amino acid sequence identity with CryIAa toxin (8).

In many cases, *in vitro* binding studies using ^{125}I-labeled toxins and midgut brush border membrane vesicles (BBMV)[1] isolated from susceptible and resistant insect larvae have shown a direct correlation between insect toxicity and binding (15, 16). Recent studies on binding kinetics suggest a two-step process (reversible and irreversible) for Cry toxins with several lepidopteran insects (17, 18). Interestingly, a direct correlation between toxicity and the rate constant for irreversibly bound toxin has been observed (17). Recent progress on the identification and purification of insect midgut toxin-binding (receptor) molecules suggest 120- and 210-kDa proteins from *Manduca sexta* as binding proteins for CryIAc and CryIAb toxins, respectively (19, 20). In gypsy moth BBMV, CryIAa, and CryIAb toxins bind to a 210-kDa protein and CryIAc binds to a 120-kDa amino peptidase-N (13, 21).

The domain II loop residues of Cry toxins have been targeted

* This work was supported by Grant RO1 29092 from the National Institutes of Health (to D. H. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: BBMV, brush boarder membrane vesicle; PAGE, polyacrylamide gel electrophoresis.

by site-directed mutagenesis and membrane binding assays to investigate the molecular basis for the action of δ-endotoxins. Wu and Dean (22) mutated the loop residues of CryIIIA and observed that loops 1 and 3 are involved in receptor binding. Recent studies by Rajamohan *et al.* (18, 23) on CryIAb showed that loop 2 residues, ^{368}RRP^{370}, are involved in initial receptor binding, while residues Phe^{371} and Gly^{374} of the same loop are largely involved in irreversible binding of the toxin to *M. sexta*. In earlier reports, deletion of a portion of CryIAa loop 2 (residues 365–371) removed nearly all toxicity and initial binding to *Bombyx mori* (24), while mutations in loop 1 showed no effect on initial binding (25). These studies establish the significance of domain II loop residues in receptor binding. In the present communication we target another loop in domain II, loop 3, of CryIAa and CryIAb toxins and analyze its functional role in receptor binding and toxicity. We demonstrate that either deletion or alanine substitution of loop 3 amino acids, especially affecting hydrophobic residues, of CryIAa and CryIAb toxins significantly affect the initial binding ability to *M. sexta*, *B. mori*, and *Heliothis virescens* membrane vesicles and reduce their potency to the target insects.

MATERIALS AND METHODS

Site-directed Mutagenesis—The oligonucleotides used for site-directed mutagenesis were kindly provided by Dr. Takashi Yamamoto, Sandoz Agro Inc., Palo Alto, CA. A uracil-containing template of *cryIAa* and *cryIAb* genes was obtained by transforming *Escherichia coli* CJ236 (Bio-Rad) with pOSM1313 (26) and pSB033b (18), respectively. The site-directed mutagenesis procedure followed the manufacturer's manual (Muta-Gene M13 *in vitro* mutagenesis kit; Bio-Rad). DNA sequencing was carried out by the method of Sanger *et al.* (27) following the manufacturer's (U. S. Biochemical Corp.) instructions. Fine chemicals and restriction enzymes were purchased from Boehringer Mannheim.

Expression and Purification of Toxin—Mutant and wild-type δ-endotoxins were expressed in *E. coli* MV1190 and were purified as described previously (18). The purified crystal protein was solubilized in crystal solubilization buffer (50 mM Na_2Co_3, pH 9.5, 10 mM dithiothreitol) at 37 °C for 3 h. Activation of the solubilized protoxin was carried out by treating with 2% (by mass) trypsin (Sigma) at 37 °C for 5 h and was analyzed by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (PAGE).

Protein Determination—Protein concentrations of toxins and BBMV were determined with the Coomassie protein assay reagent (Pierce) using bovine serum albumin as a standard.

Protease Digestions and Western Blotting—Insect gut enzyme digestion of mutant and wild-type proteins was performed by incubating the toxin with freshly prepared gut enzymes at 37 °C for 3 h as described before (18). The final digested products were separated on SDS-10% PAGE, transferred onto polyvinylidene difluoride membrane (Bio-Rad); treated with anti-CryIAa or CryIAb serum and the blot was processed and developed as described previously (18).

Toxin Iodination—Twenty μg of trypsin-activated toxin were labeled using 1 mCi of ^{125}I (Dupont) and one IODOBEAD (Pierce) following the manufacturer's directions. Free iodine was separated from the toxin using a prepacked Excellulose GF-5 column (Pierce) as described previously (18). The specific activities of labeled CryIAa, A3a, D3a, CryIAb, S438A, G439A, F440A, S441A, N442A and S443A were 1.9, 1.9, 1.7, 2.1, 1.9, 2.3, 1.4, 1.5, 1.8, and 1.6 mCi/mg, respectively.

Preparation of BBMV—Insect midguts were prepared as described previously (12) and were stored at liquid nitrogen or at −70 °C until use. BBMV were prepared by the differential magnesium precipitation method as modified by Wolfersberger *et al.* (28). The final vesicles were resuspended in binding buffer (8 mM $NaHPO_4$, 2 mM KH_2PO_4, 150 mM NaCl, pH 7.4, containing 0.1% bovine serum albumin) to a final protein concentration of 1 mg/ml and stored at −70 °C until use.

Toxicity Assays—*B. mori* eggs were kindly supplied by R. E. Milne, Forest Pest Management Institute, Sault Ste. Marie, Canada. The eggs were hatched and raised to third instar on mulberry leaves. A 5.0-μl total volume of each toxin dilution (diluted in phosphate-buffered saline, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, 150 mM NaCl, pH 7.4) was delivered into the larval midgut with a 0.25-ml syringe (Yale-BD) fitted with a blunted and polished 30-gauge needle, using a model 1003 Microjector syringe drive and a model 1010 Microdoser (Houston Atlas, Houston, TX). Three sets of 10 larvae were used for each toxin concentration (at least five toxin concentrations were used per toxin), and the dosed larvae were then fed on mulberry leaves. Control insects were dosed with the same volume of phosphate-buffered saline, and the toxicity was assessed after 24 h. Effective dose estimates were obtained by Probit analysis (29).

M. sexta eggs used in this study were supplied by D. L. Dahlman (Dept. of Entomology, University of Kentucky, Lexington). The eggs were hatched and raised on artificial diet (Bio Serve). Toxicity assays were performed with newly hatched larvae as described in Rajamohan *et al.* (23). The mortality rates were recorded after 5 days, and the results were analyzed using probit analysis (29).

The *H. virescens* strain (YDK) used in this study was described in Gould *et al.* (30). Bioassay and data analysis procedures were performed as described previously by Rajamohan *et al.* (23).

Competition Binding Assay—Homologous (competition between labeled and nonlabeled forms of the same ligand) and heterologous (competition between one labeled ligand and another nonlabeled ligand) competition binding assay procedures were as described elsewhere (18). In short, 100 μg/ml BBMV were incubated with 1 nM of labeled toxin and increasing concentrations (0 to 1000 nM) of appropriate nonlabeled toxin in 100 μl of binding buffer at room temperature for 1 h. The pellet was washed three times with binding buffer to remove the unbound toxins, and the radioactivity in the final pellet was measured in a gamma counter (Beckman). Each experiment was repeated at least three times, and the mean values were plotted using the CA-CRICKET Graph III application program. A meticulous kinetic binding study of CryI toxins performed by Liang *et al.* (17) pointed out the inappropriate use of the term K_d (dissociation binding constant), which has been used in previous studies for competition binding of Cry toxins with BBMV (11, 12, 14, 17). In a later study, Wu and Dean (22) used an alternative term, K_{com} for binding constants calculated from competition studies of Cry toxins with BBMV using the LIGAND program (31). Hence, in this study, the term K_{com} will be used in place of K_d. The binding affinity (K_{com}) and binding site concentrations (B_{max}) were calculated using the LIGAND program (31).

Dissociation Binding Assay—Dissociation binding assays were performed essentially as described previously (18). 1.0 nM ^{125}I-labeled toxin was incubated with 200 μg/ml BBMV for 1 h at room temperature (association binding) to achieve saturation binding. Nonlabeled toxin (100 nM, final concentration, in a 20-μl volume) was then added to each sample tube, and the reaction was stopped at different time intervals (5 min to 1 h) by centrifugation. The radioactivity in the pellets was measured in a gamma counter (Beckman).

Voltage Clamp Analysis—The dissection and mounting of *M. sexta* midgut followed the protocol by Harvey *et al.* (32). The amplifier equipment for voltage clamp consisted of a D.C. 1000 voltage/current clamp, an A-310 Accupulser (World Precision Instruments), and a strip chart recorder (Kipp and Zonen). The voltage clamp analysis was performed as described by Rajamohan *et al.* (23). After stabilization of the membrane, trypsin-activated toxin was injected into the lumen side of the gut (final concentration, 50 ng/ml). The I_{sc} (inhibition of short-circuit current) was tracked with a recorder, and data were collected with the MacLab data acquisition system. Each individual experiment was repeated at least three times, and the mean values were plotted using the CA-CRICKET Graph III application program.

RESULTS

Alignment and Construction of Mutants—The alignment of domain II, loop 3, residues of CryIAa toxin (8), with CryIAb toxin are shown in Fig. 1*A*. Deletion and alanine substitution mutations constructed at the loop 3 region of CryIAa and alanine substitutions of CryIAb loop 3 are shown in Fig. 1*B*.

Expression and Stability of Mutant Proteins—The δ-endotoxin inclusion bodies purified from wild-type and mutants were solubilized and analyzed on SDS-10% PAGE. All the mutant proteins were expressed in amounts comparable to wild-type (Figs. 2*B* and 3*A*). Each also yielded a 60-kDa stable, trypsin-resistant toxin core upon activation with trypsin (Figs. 2*A* and 3*B*). To investigate the stability of trypsin-activated toxins with insect gut proteases, the toxins were treated further with gut juice collected from target insects. Western blot analysis showed that all CryIAb (Fig. 2) and CryIAa (Fig. 3) mutant proteins yielded stable 60-kDa toxin, similar to wild-type, upon treatment with *M. sexta* (Figs. 2*C* and 3*D*), *H. virescens* (Fig. 2*D*), and *B. mori* (Fig. 3*C*), gut juice.

Toxicity and Binding of CryIAb Mutants to M. sexta—The LC_{50}, K_{com}, and B_{max} values of CryIAb and mutants S438A, G439A, F440A, S441A, N442A, and S443A toward *M. sexta* were analyzed, and the values are reported in Table I. Mutants G439A and F440A reduced the toxicity (100 and 20 times less, respectively) to *M. sexta*. The LC_{50} values of mutants S438A, S441A, N442A, S443A, and wild-type were 45.5, 10.8, 37.3, and 43.6, and 9.7 ng/cm^2, respectively. These mutants were similar or up to 4 times less potent when compared to the wild-type toxin. The K_{com} of mutants G439A and F440A was 12 and 9 times, respectively, higher than that of CryIAb (Table I). The heterologous binding curves showed that mutant toxins G439A and F440A competed for the binding of labeled wild-type toxin with reduced binding affinity compared to CryIAb, S438A, S441A, N442A, and S443A toxins (Fig. 4*A*). Dissociation binding assays with CryIAb, G439A, and F440A labeled toxins showed that about 85–90% of the BBMV-bound toxins were irreversibly associated with the vesicles. However, the total amount of toxin that irreversibly bound to the BBMV was significantly different between the wild-type and the mutants. While 38 ng/mg BBMV of CryIAb toxin was irreversibly associated with the vesicles, only 18 and 15 ng/mg BBMV of F440A and G439A toxins, respectively, were irreversibly bound to *M. sexta* vesicles (Fig. 5).

Response of M. sexta Midgut to CryIAb Mutant Toxins—The voltage clamp experiment measures the active transport of ions across the midgut cells from the hemolymph side to the lumen side. The inhibition of short-circuit current (I_{sc}) illustrates the depolarization of the midgut membrane due to the channel forming activity of Cry toxin. Our experiments showed that the slope of I_{sc} inhibition of CryIAb, S438A, S441A, N442A, and S443A toxins were between −94 to −97 μA/cm^2/min (Table I), whereas the slope for F440A was −60.3 μA/cm^2/min. We were unable to calculate the slope of mutant G439A because of insufficient inhibition of I_{sc} by the mutant toxin at this concentration (Fig. 6).

Binding and Toxicity of CryIAb Mutants to H. virescens—The biological activity of CryIAb and mutant toxins to *H. virescens* were compared and reported in Table II. The LC_{50} of S438A, S441A, N442A, and S443A showed that these mutants were only 2–4 times less toxic (LC_{50} 3.6, 1.6, 1.2, and 2.2 μg/ml diet, respectively) than the wild-type (LC_{50} 0.82 μg/ml diet). In contrast, G439A lost most of its toxicity (insufficient mortality at 15 μg/ml concentration to calculate the exact LC_{50}), and F440A reduced the toxicity 15 times compared to wild-type (Table II). The K_{com} estimated by homologous competition assays for wild-type, S438A, S441A, N442A, and S443A toxins were between 3.17 and 5.9 nM (Table II), whereas G439A and F440A were 6–7 times higher (22.33 and 19.97 nM, respectively). In heterologous competition binding studies CryIAb, S438A, S441A, N442A, and S443A toxins competed for binding with higher affinity to *H. virescens* BBMV (Fig. 4*B*). Mutant toxins G439A and F440A competed with reduced binding affinity (the binding curves were shifted to the right) for the binding sites of labeled wild-type toxin as shown in Fig. 4*B*. The dissociation binding data with *H. virescens* were similar to that of *M. sexta* reported here, and CryIAb toxin bound 3.7 times more than the mutants G439A and F440A (data not shown).

Insect Bioassay and Binding of CryIAa Mutants—The toxic-

A

β10 **Loop 3** β11

CryIAa $_{428}$SHRLSHVTMLS[redacted]TLRAPTFSWQHRSAE$_{460}$

CryIAb $_{427}$SHRLSHVSMFRSGFSNSSVSIIRAPMFSWIHRSAE$_{461}$

B

Mutants:

CryIAa

D3a (440-443) $_{428}$SHRLSHVTMLSQ----VYTLRAPTFSWQHRSAE$_{460}$

A3a (437-439) $_{428}$SHRLSHVTMAAAAAGAVYTLRAPTFSWQHRSAE$_{460}$

CryIAb

S438A $_{427}$SHRLSHVSMFRAGFSNSSVSIIRAPMFSWIHRSAE$_{461}$

G439A $_{427}$SHRLSHVSMFRSAFSNSSVSIIRAPMFSWIHRSAE$_{461}$

F440A $_{427}$SHRLSHVSMFRSGASNSSVSIIRAPMFSWIHRSAE$_{461}$

S441A $_{427}$SHRLSHVSMFRSGFANSSVSIIRAPMFSWIHRSAE$_{461}$

N442A $_{427}$SHRLSHVSMFRSGFSASSVSIIRAPMFSWIHRSAE$_{461}$

S443A $_{427}$SHRLSHVSMFRSGFSNASVSIIRAPMFSWIHRSAE$_{461}$

FIG. 1. *A*, alignment of the toxin protein sequences of CryIAa with CryIAb between residues 427 and 461. The positions of the secondary structural elements of CryIAa are given above its sequence as described by Grochulski *et al.* (8). The loop 3 residues are highlighted. *B*, summary of deletion and alanine substitutions constructed on CryIAa and CryIAb toxins. *A3a*, alanine substitution of CryIAa residues ^{437}LSQ^{439}; *D3a*, deletion of CryIAa residues ^{440}AAGA^{443}.

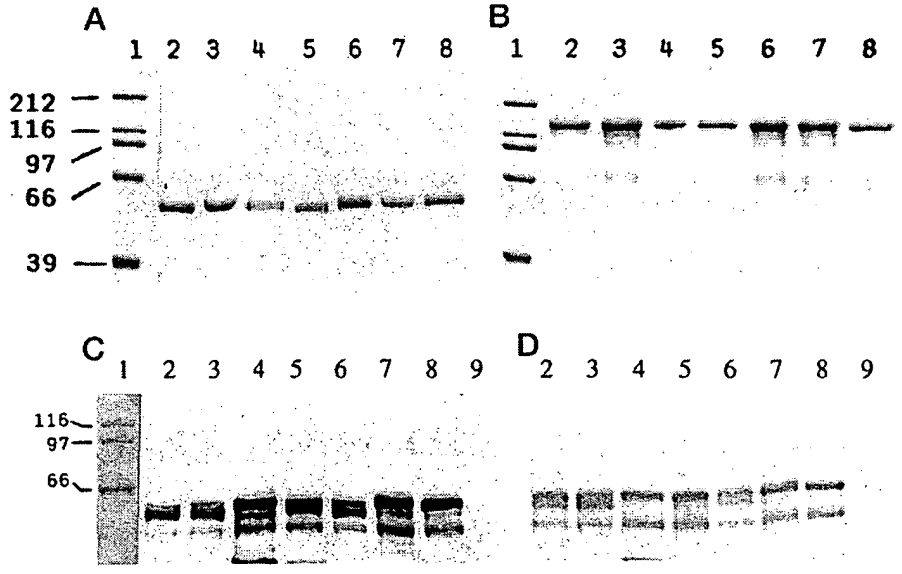

FIG. 2. **Coomassie blue-stained SDS-10% PAGE of CryIAb mutants, comparing the yield of trypsin-activated toxins (*A*) protoxins (*B*), Western blot analysis of the stability of wild-type and mutant proteins after digestion with *M. sexta* (*C*) and *H. virescens* (*D*) gut juice.** *Lane 1*, molecular mass markers. Masses of the protein markers (in kilodaltons) are shown on the left; *lane 2*, CryIAb; *lane 3*, S438A; *lane 4*, G439A; *lane5*, F440A; *lane 6*, S441A; *lane 7*, N442A; *lane 8*, S443A; and *lane 9*, corresponding insect gut juice.

ity of CryIAa and loop 3 mutant toxins toward *B. mori* and *M. sexta* were analyzed and reported in Table III. The LC_{50} values of A3a to *M. sexta* and *B. mori* (3.1 and 46.3 ng, respectively) were similar to the wild-type toxin (2.5 and 40.5 ng, respectively). Whereas the mutant D3a is about 28 times less toxic to *M. sexta* and >62 times less toxic to *B. mori* when compared to the wild-type toxin (Table III).

The binding affinity (K_{com}) and binding site concentrations (B_{max}) of CryIAa and mutant toxins to midgut vesicles prepared from *B. mori* and *M. sexta* were calculated by homologous competition binding assays, and the results were shown in Table III. The K_{com} and B_{max} value of the mutant A3a was comparable with CryIAa for both the insects, whereas the K_{com} value of D3a was about 15 and 9 times higher than CryIAa, for *M. sexta* and *B. mori*, respectively, representing reduced binding affinity (Table III). When the wild-type toxin was labeled with ^{125}I and put into competition with nonlabeled wild-type or mutant toxins, CryIAa and A3a displayed higher affinity binding to *B. mori* and *M. sexta* (Fig. 7, *A* and *B*), whereas the D3a curve was shifted to the right compared with that of CryIAa or A3a to both insect BBMV (Fig. 7, *A* and *B*).

DISCUSSION

Elucidation of the mechanism of interaction between the δ-endotoxin and insect midgut receptor(s) is critical to the rational design of improved insecticidal toxins with broader insect specificity and higher larvicidal potency. The insect specificity determining region of CryI type toxins has been located primarily in domain II for several lepidopterans (26, 33, 34). The three-dimensional structure of CryIAa suggests that the apex of domain II is composed of three solvent-exposed loops comprising residues 310–313 (loop 1), 367–379 (loop 2), and 438–446 (loop 3). The striking dissimilarity between CryIAa and CryIAb toxins in amino acid sequences in loops 2 and 3 inspired us to investigate the role of these residues in insect specificity, toxicity, and receptor binding.

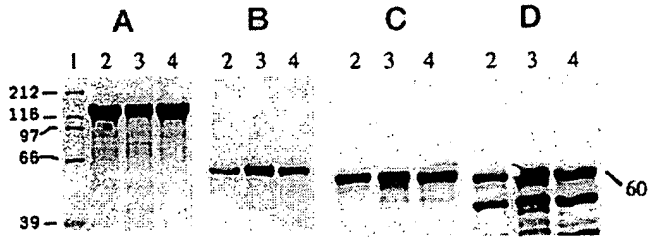

FIG. 3. **Coomassie blue-stained SDS-10% PAGE of CryIAa mutants, comparing the yield of protoxins (*A*) trypsin-activated toxins (*B*), Western blot analysis of the stability of wild-type and mutant proteins after digestion with *B. mori* (*C*) and *M. sexta* (*D*) gut juice.** *Lane 1*, molecular mass markers. Masses of the protein markers (in kilodaltons) are shown on the left; *lane 2*, CryIAa; *lane 3*, A3a; and *lane 4*, D3a.

Although CryIAa and CryIAb share 89% overall amino acid sequence identity and bind to the same receptor (210 kDa) in the gypsy moth, loop 3 residues of domain II are significantly different (Fig. 1). In this study, loop 3 residues, ^{438}SGFSNS^{443}, of CryIAb toxin were individually replaced with alanine and tested for toxicity to *M. sexta* and *H. virescens*. Our bioassay

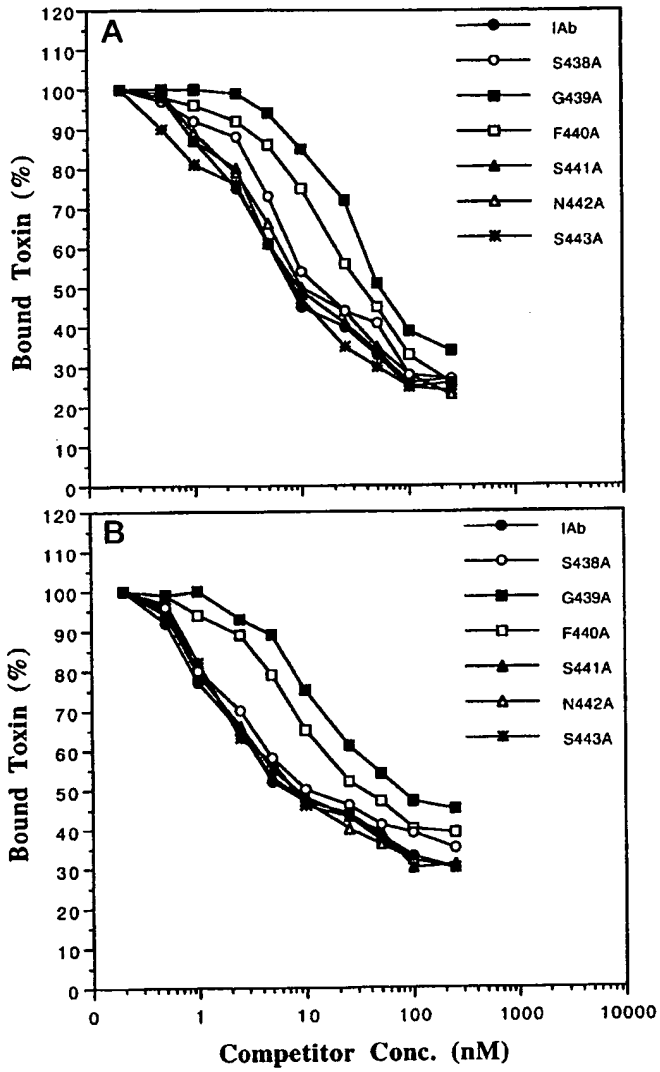

FIG. 4. **Binding of ^{125}I-labeled (1 nM) CryIAb toxin in the presence of increasing concentrations of nonlabeled CryIAb, S438A, G439A, F440A, S441A, N442A, and S443A toxins to *M. sexta* (*A*) and *H. virescens* BBMV (*B*).** Binding is expressed as a percentage of the total amount bound upon incubation with labeled toxin alone. On *M. sexta* and *H. virescens* vesicles, the amount is 2400 ± 80 cpm and 2250 ± 35 cpm, respectively for CryIAb.

TABLE I

Insecticidal activity, binding affinity, and I_{SC} inhibition of CryIAb mutant proteins to M. sexta

Toxins	LC_{50}[a]	K_{com}[b]	B_{max}[c]	I_{SC} inhibition rate[d]
	ng/cm²	*nM*	*pmol/mg*	*μA/cm²/min*
CryIAb	9.7 (6.2–14)	1.8 ± 0.55	4.05 ± 0.4	−97.4 ± 5.6
S438A	45.5 (37–61)	2.77 ± 0.71	5.25 ± 0.2	−93.1 ± 4.9
G439A	1000 (920–1300)	21.0 ± 2.70	9.05 ± 1.1	UD[e]
F440A	190 (155–222)	16.1 ± 3.11	8.11 ± 1.7	−60.3 ± 4.1
S441A	10.8 (6.5–16)	1.9 ± 0.31	4.91 ± 0.7	−94.1 ± 5.2
N442A	37.3 (26–51)	3.0 ± 0.71	4.99 ± 0.8	−93.9 ± 8.9
S443A	43.6 (36–53)	2.69 ± 0.84	5.79 ± 0.7	−94.3 ± 3.1

[a] LC_{50}, 50% lethal concentration, 95% confidence intervals are given in parentheses.
[b] K_{com}, dissociation constant (determined from homologous competition binding). Each value is the mean of three individual experiments.
[c] B_{max}, binding site concentration (determined from homologous competition binding). Each value is the mean of three experiments.
[d] Each value if the mean of three experiments.
[e] Undetectable.

data show that the mutants G439A (alanine replacement of Gly^{439}) and F440A have substantially reduced toxicity to *M. sexta* (100 and 20 times, respectively) and *H. virescens* (>15 times). The other mutants (S438A, S441A, N442A, and S443A) only marginally affected toxicity. Evidence such as 1) expression of mutant toxins at levels comparable to wild-type (Fig. 2*B*), 2) stability of the mutant toxins (60 kDa) upon digestion with trypsin (Fig. 2*A*), and 3) processing wild-type and mutant toxins alike into 60-kDa toxin by insect gut enzyme digestion (Fig. 2, *C* and *D*) suggest that the loss of toxicity was not caused by instability of mutant toxins in the insect midgut. It was noticed that the digestion of mutant toxins, especially G439A, with *M. sexta* and *H. virescens* gut juice generated a few minor peptides in addition to the stable 60-kDa toxin, suggesting that G439A might be slightly more susceptible to insect gut proteases than the wild-type. However, we do not expect this to account for the substantial loss of toxicity of G439A (100 and >12 times for *M. sexta* and *H. virescens*, respectively) to the test insects, since these minor peptides were also observed in the mutants (S441A, N442A, and S443A) which are relatively as toxic as the wild-type. Competition binding assays revealed that the K_{com} of wild-type and toxic mutants (S438A, S441A, N442A, and S443A) did not differ for either of the insect (Tables I and II). In contrast, the K_{com} of G439A and F440A were considerably higher than wild-type with *M. sexta* (12 and 9 times, respectively) and *H. virescens* (7 and 6 times, respectively), suggesting lower binding affinity of these mutants to midgut membrane vesicles (Fig. 4, *A* and *B*). These results correlate with our bioassay data that toxins with higher potency (CryIAb, S438A, S441A, N442A, and S443A) exhibit higher affinity binding, whereas toxins with marginal potency (G439A and F440A) show weaker binding. Our dissociation binding assays showed that alanine substitution of Gly^{439} and Phe^{440} did not affect the off-rate of the BBMV-bound toxin since 85–90% of bound wild-type and mutants (G439A and F440A) were irreversibly associated to the midgut vesicles prepared from both target insects. By comparison to wild-type toxin in both target insects, mutants G439A and F440A showed 3.5-fold reduction in the amount bound (Fig. 5). The marginal potency in bioassay exhibited by mutants G439A and F440A is consistent with the initial binding analyses showing that midgut vesicles from both target insects show reduced numbers of mutant toxins bound as well as lowered binding affinity. We also examined the inhibition of I_{sc} across the isolated midgut in response to the addition of wild-type and mutant toxins. The toxins that have higher binding affinity to the receptor inhibit the I_{sc} more efficiently than the toxins with reduced binding affinity (Fig. 6).

The involvement of CryIAb, loop 3 Gly residue (Gly^{439}) in initial receptor binding toward *H. virescens* is in agreement with our previous observation that when loop 2, Gly^{374}, was mutated to Ala, it reduced the initial binding to *H. virescens* BBMV (23). The loss of toxicity of G439A could be argued as a result of minor changes in the flexibility of the loop, since Gly promotes turns and mobility of loops (35). Therefore, it may

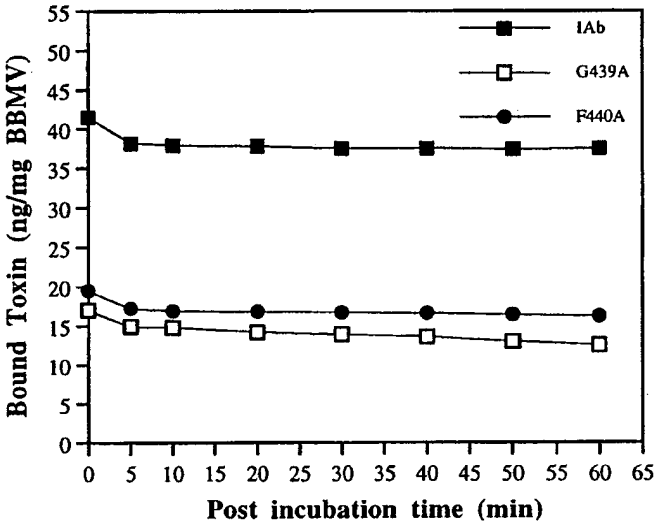

FIG. 5. **Dissociation of bound ^{125}I-labeled toxins from *M. sexta* BBMV.** *M. sexta* BBMVs (200 μg/ml) were incubated with 1 nM ^{125}I-labeled CryIAb, G439A, and F440A toxins for 1 h (association reaction). After the association reaction, 100 nM corresponding nonlabeled toxins were added to the test samples, and incubation was continued (post-binding incubation). Binding is expressed as nanograms of toxin bound/mg of BBMV.

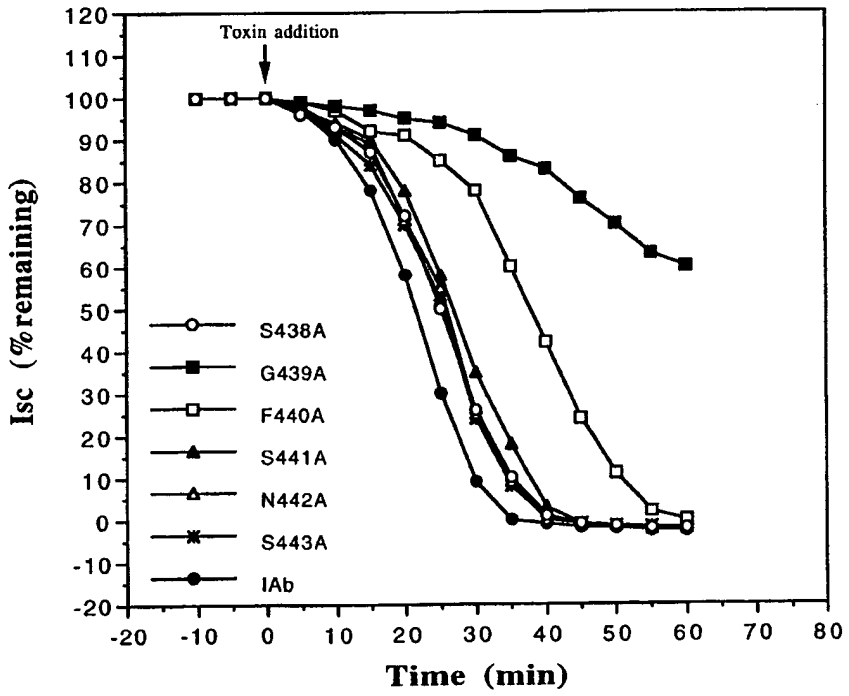

FIG. 6. **Inhibition of I_{sc} across *M. sexta* midgut.** A total of 50 ng/ml CryIAb, S438A, G439A, F440A, S441A, N442A, and S443A toxin were injected in separate experiments into the lumen side of the chamber, and the drop in I_{sc} was measured. The I_{sc} measured before the addition of the toxin is considered as 100%.

TABLE II
Biological activity and binding affinity of CryIAb mutant proteins toward H. virescens

Toxin	LC_{50}[a]	K_{com}[b]	B_{max}[c]
	μg/ml	*nM*	*pmol/mg*
CryIAb	0.82 (0.4–1.5)	3.17 ± 0.21	30.51 ± 3.5
S438A	3.61 (2.2–6.2)	4.21 ± 0.80	34.25 ± 3.1
G439A	>15	22.33 ± 2.31	42.01 ± 2.7
F440A	12.44 (6.4–26)	19.97 ± 3.11	44.91 ± 3.3
S441A	1.6 (0.9–2.9)	5.91 ± 0.27	29.39 ± 2.8
N442A	1.2 (0.5–5.3)	3.01 ± 0.99	31.66 ± 1.9
S443A	2.2 (1.3–3.6)	4.99 ± 1.11	28.98 ± 2.6

[a] LC_{50}, 50% lethal concentration, 95% confidence intervals are given in parentheses.
[b] K_{com}, dissociation constant (determined from homologous competition binding). Each value is the mean of three individual experiments.
[c] B_{max}, binding site concentration (determined from homologous competition binding). Each value is the mean of three experiments.

TABLE III
Insecticidal activity and binding constants of CryIAb mutant proteins to M. sexta and B. mori

Toxin	LC_{50}[a]	K_{com}[b]	B_{max}[c]
	ng/cm²	*nM*	*pmol/mg*
M. sexta			
CryIAb	2.5 (1.1–4.5)	1.17 ± 0.5	20.51 ± 3.5
A3a	3.1 (1.5–5.1)	3.21 ± 0.8	24.25 ± 3.9
D3a	70.5 (61–89)	10.30 ± 1.3	110.0 ± 7.7
B. mori	LD_{50}[d] (*ng/larvae*)		
CryIAa	40.5 (32.1–51.8)	0.8 ± 0.05	15.25 ± 3.4
A3a	46.3 (39.1–55.7)	1.77 ± 0.2	21.25 ± 4.1
D3a	>2,500	12.0 ± 1.7	95.05 ± 6.8

[a] LC_{50}, 50% lethal concentration, 95% confidence intervals are given in parentheses.
[b] K_{com}, dissociation constant (determined from homologous competition binding). Each value is the mean of three individual experiments.
[c] B_{max}, binding site concentration (determined from homologous competition binding).
[d] LD_{50}, 50% lethal dose, 95% confidence intervals are given in parentheses. Each value is the mean of three experiments.

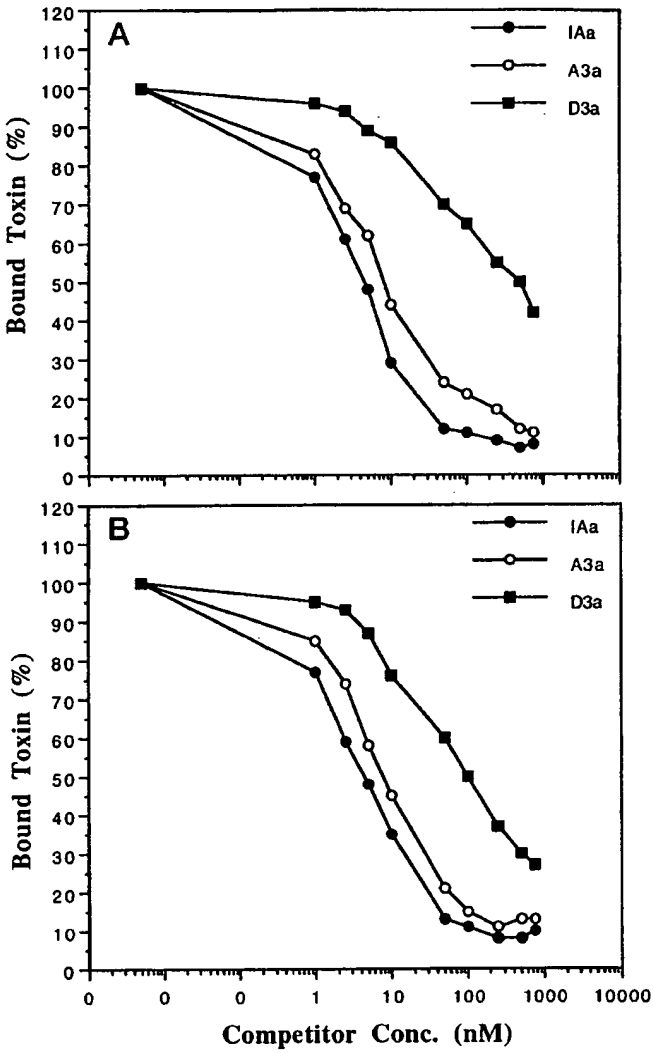

FIG. 7. **Binding of ^{125}I-labeled (1 nM) CryIAa toxin in the presence of increasing concentrations of nonlabeled CryIAa, A3a, and D3a toxins to *B. mori* BBMV (*A*) and *M. sexta* BBMV (*B*).** Binding is expressed as a percentage of the total amount bound upon incubation with labeled toxin alone. The amount for CryIAa is 1380 ± 50 cpm and 2100 ± 45 cpm on *B. mori* and *M. sexta* vesicles, respectively.

indirectly affect binding affinity. Phe at loop 3 plays a critical role in toxicity and receptor binding, similar to the role in loop 2 (18), but its effect on binding is significantly different in the two loops. Alanine substitution of Phe^{371} at CryIAb loop 2 did not have any effect on initial receptor binding, but extensively affected the irreversible association of the toxin to the BBMV and dramatically reduced (400 times less) the toxicity to *M. sexta* (18). In contrast, alanine substitution of Phe^{440} (a stronger hydrophobic residue than alanine) in the loop 3 affected the initial binding of the toxin to the same insect. This may suggest that Phe plays functionally distinct roles (initial binding and irreversible binding to *M. sexta*) when located at different loops of CryIAb toxin. Furthermore, alanine substitution of two positively charged residues at loop 2 (^{368}RR^{369}) eliminates the initial binding of the toxin almost completely with *M. sexta* BBMV (23). Considering these results, we propose that the initial receptor recognition process of the toxin is a combination of charge (loop 2) and hydrophobic (loop 3) interactions.

To further examine the functional role of hydrophobic residues, we mutated the loop 3 residues of CryIAa. We have constructed two mutants, A3a (alanine substitution of residues ^{437}LSQ^{439}) and D3a (deletion of ^{440}AAGA^{443}). Digestions with trypsin and insect (*B. mori* and *M. sexta*) gut juice enzyme (the ultimate environment that determines the stability of the toxin) showed that the mutant proteins were as stable as the wild-type toxin (Fig. 3, *B–D*). Further studies with A3a on insect toxicity and binding revealed that this mutant affected neither toxicity nor binding for *B. mori* and *M. sexta*, which are highly susceptible to CryIAa toxin (Table III, Fig. 7, *A* and *B*).

Since the residues ^{437}LSQ^{439} are located between β10 and loop 3, they might not be completely exposed for the interaction with the receptor. On the contrary, D3a reduced the toxicity >68 times to *B. mori* and 28 times to *M. sexta* (Table III). Binding experiments with insect midgut vesicles showed that the deletion of relatively hydrophobic loop residues ^{440}AAGA^{443} (D3a) disrupted binding affinity (K_{com}) by 15 times to *B. mori* and 9 times to *M. sexta* (Table III). Hence, it is reasonable to speculate that the reduced potency of D3a to both insects is attributable to the reduced initial binding affinity. These experiments suggest that the loop 3 residues (440–443) of CryIAa contain important binding determinants to *B. mori* and *M. sexta* receptor(s). Considering the lack of any active side chain among the loop 3 residues, ^{440}AAGA^{443}, the stretch of alanines might provide hydrophobicity, and their deletion could remove a hydrophobic interaction. Consequently, the studies with CryIAa also support our earlier proposal that the hydrophobic residues of loop 3 are important for initial receptor binding. We have previously reported that a deletion of charged and hydrophobic loop 2 residues (^{365}LYRRIIL^{371}) of CryIAa resulted in substantial loss of initial binding and toxicity to *B. mori* (24). It is obvious from these studies that in both CryIAa and CryIAb

toxins charge (loop 2) and hydrophobicity (loops 2 and 3) play a key role in initial receptor binding.

Our experiments do not exclude the possibility that the toxins (CryIAa and CryIAb) bind to two different binding sites on the same receptor, one with higher binding affinity and the other with lower affinity. In that case, the loop 3 mutations selectively affected a higher affinity site that is important for toxicity. These results provide evidence that the receptor binding residues of these toxins are physically scattered, rather than clustered in a confined region of the toxin. Recent studies with domain swapping experiments suggest that domain III, in addition to domain II, is involved in insect specificity and receptor recognition (13, 36). As a result, these loops are excellent targets for genetic redesigning of novel toxins with diverse specificity by exchanging the residues or chain lengths of the active site without affecting the structural frame work of the toxin.

Acknowledgments—We thank Daniel R. Zeigler, Mi K. Lee, Oscar Alzata, and S. J. Wu for their critical evaluation and Dr. Takashi Yamamoto (Sandoz Agro Inc.) for preparing the mutagenic oligonucleotides. We are grateful to Dr. David Stetson (Department of Zoology, The Ohio State University) for the use of the voltage clamp apparatus.

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FEBS Letters 497 (2001) 108–112 FEBS 24872

Mutations at the arginine residues in α8 loop of *Bacillus thuringiensis* δ-endotoxin Cry1Ac affect toxicity and binding to *Manduca sexta* and *Lymantria dispar* aminopeptidase N

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Received 1 March 2001; revised 19 April 2001; accepted 26 April 2001

First published online 9 May 2001

Edited by Pierre Jolles

Abstract The functional role of the α8 loop residues in domain II of *Bacillus thuringiensis* Cry1Ac toxin was examined. Alanine substitution mutations were introduced in the residues from 275 to 293. Among the mutant toxins, substitutions at R281 and R289 affected toxicity to *Manduca sexta* and *Lymantria dispar*. Loss of toxicity by these mutant toxins was well correlated with reductions in binding affinity for brush border membrane vesicles and the purified receptor, aminopeptidase N (APN), from both insects. These data suggest that the two arginine residues in the α8 loop region are important in toxicity and APN binding in *L. dispar* and *M. sexta*.

Key words: Brush border membrane vesicle; Aminopeptidase N; Surface plasmon resonance; *Bacillus thuringiensis*; *Lymantria dispar*; *Manduca sexta*

1. Introduction

Despite its importance as a systemic pesticide in genetically modified plants, the mechanism of action of *Bacillus thuringiensis* (Bt) δ-endotoxin remains unclear. It is generally accepted that Bt crystals are ingested by susceptible insect larvae, solubilized in the midgut, and simultaneously digested to active toxins by midgut proteases. Activated toxins bind to specific receptor molecules present in the midgut epithelial cell brush border membrane and insert into the membrane, forming a pore or ion channel that ultimately kill the insect. The details of how Bt toxins associate with receptors and insert into membranes is still an area of active research and controversy.

Solution of the X-ray crystal structures of Cry3A and Cry1Aa revealed a conserved three-domain composition of Bt toxins [1,2]. Site-directed mutagenesis has been conducted extensively within each domain to localize regions responsible for toxicity, receptor binding and pore formation. Briefly, domain I contains seven α-helices and is believed to be responsible for membrane spanning and pore formation. Site-directed mutagenesis in the helices altered toxicity by changing pore-forming activity. Domains II and III are composed almost exclusively of β-sheets and turns. Domain II was originally proposed to be a receptor-binding domain. Mutations in the loop regions (α8, loop 1, 2, 3) of domain II affected toxicity by changing reversible or irreversible BBMV binding. Domain switches and site-directed mutagenesis of domain III demonstrated that domain III is important in specificity, receptor binding, and pore formation [3].

In order to understand toxicity, specificity, and in some cases, insect resistance mechanisms, the receptor-binding properties of Bt toxins have been extensively examined using brush border membrane vesicles (BBMV) [4–6]. To directly examine toxin receptor-binding properties, several Bt toxin receptors have been identified. A Cry1A toxin-binding protein, 120-kDa aminopeptidase N, has been identified and purified from *Manduca sexta*, *Lymantria dispar*, and other insects and its gene was cloned [7–11]. A 210-kDa cadherin-like molecule was also identified as a Cry1A toxin receptor [12]. Recently, interaction between Bt toxin and purified receptor has been studied using surface plasmon resonance (SPR) [13–17]. To *M. sexta* APN, Cry1Ac binds to two sites with 2:1 toxin receptor stoichiometry. One site requires only domain III, whereas the other requires both domain III and domain II for complete binding [16,18]. However, it has been demonstrated that domain III binding of *M. sexta* APN does not correlate with toxicity [19–21], but domain II binding does [22]. There is evidence that domain II of Cry1Ac is necessary for binding not only APN, but also the 210-kDa receptor [16].

In *L. dispar*, Cry1Ac binds APN with a 1:1 stoichiometry. A recent study on the binding of Cry1Ac to the *L. dispar* APN proposed that a cavity in lectin-like domain III initiates docking through recognition of an *N*-acetylgalactosamine moiety on APN, then a higher affinity binding occurs through domain II [17]. Like *M. sexta*, it was observed that domain II mutations had a profound effect on insecticidal activity compared to domain III mutations. These findings were further supported by mutations at R368 and R369 of Cry1Ac domain II, which dramatically affected toxicity, BBMV binding, and APN binding in *M. sexta* and *L. dispar* [22].

A previous study showed that the mutations in the α8 loop of domain II of Cry1Ab affected toxicity and BBMV binding to *L. dispar*. Decreased activity was directly correlated with the reduced binding affinity for BBMV [23]. In the present

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0014-5793/01/$20.00
PII: S0014-5793(01)02446-2

study, the functional role of α8 loop of Cry1Ac toxin is examined. Alanine substitutions were made at the residues from 275 to 293 and the biological activities against *M. sexta* and *L. dispar* were determined. BBMV-binding assays and SPR-binding assays with aminopeptidase N purified from both insects were also performed. Two arginine residues in the α8 loop region were found to be important in binding to BBMV and APN as well as insecticidal activity against both lepidopteran species.

2. Materials and methods

2.1. Cry1Ac mutant construction and toxin purification

The *cry1Ac1* gene (pOS4201) was subcloned into pBluescript KS+ (pOS11200) and expressed in *Escherichia coli* MV 1190. Cry1Ac mutants were constructed as described previously [24]. Inclusion bodies were purified and solubilized in 50 mM sodium carbonate buffer, pH 9.8, containing 10 mM DTT. The solubilized protoxin was digested with 2% trypsin (Sigma) at 37°C for 2 h. An additional dose of 1% trypsin was added and further incubated for 2 h. Protein concentration of protoxins and toxins was estimated by Coomassie protein assay reagent (Pierce), and the purity was examined by 10% SDS–PAGE. Toxin was further purified using a size-exclusion Superdex 200 column on an AKTA Explorer (Pharmacia Biotech AB, Uppsala, Sweden). Toxin was eluted with 20 mM phosphate buffer, pH 7.4, at 1 ml/min flow rate, or 10 mM HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl and 3.4 mM EDTA) for BIAcore studies. CD spectra analysis of toxins was conducted as previously described [19].

2.2. Insect bioassays

L. dispar eggs were kindly supplied by Gary Bernon (US Department of Agriculture, Otis Methods Development Center, Beltsville, MD, USA). *M. sexta* eggs were kindly supplied by D.L. Dahlman (Dept. of Entomology, University of Kentucky, Lexington, KY, USA). Eggs were hatched and reared on artificial diet (Bio-serv, Frenchtown, NJ, USA). Activities of toxins were determined with 2–3-day-old *L. dispar* and *M. sexta* larvae by the surface contamination method as described [25]. Toxins were diluted in 50 mM sodium carbonate buffer (pH 9.5), and 35-μl samples were applied per well (2 cm^2) on artificial diet in 24-well tissue culture plates. Two larvae were placed in each well and the mortality was recorded after 5 days. Bioassays were repeated at least five times. The effective dose estimates (LC_{50}, 50% lethal concentration of toxin) were calculated using PROBIT analysis [26].

2.3. BBMV-binding assays

BBMV was prepared from the last instar larval midguts of *L. dispar* and *M. sexta* by the magnesium precipitation method [27]. 20 μg of Cry1Ac toxin was iodinated with 1 mCi of $Na^{125}I$ (Dupont) and an IODO-BEAD (Pierce). Labeled toxin was separated from the free iodine using an Excellulose GF-5 column (Pierce). Homologous and heterologous competition binding assays were performed as described previously (MolMicro paper, 2000). K_{com} (nM) and B_{max} (pmol/mg of BBMV) values were calculated with the computer program LIGAND [28]. K_{com} represents the binding affinities calculated from BBMV competition binding experiments. This term is used to reflect the lack of steady state binding kinetics due to the irreversible membrane insertion event [29].

2.4. SPR binding assays

Binding of toxin to aminopeptidase N was studied using BIAcore®2000 with CM5 sensor chips (BIAcore AB, Uppsala, Sweden). APNs were purified from *M. sexta* and *L. dispar* BBMVs using chromatographic methods as previously described [16,17]. APN diluted in 20 mM ammonium acetate buffer, pH 4.1, was immobilized onto the carboxymethylated dextran surface of a CM 5 sensor chip using amine coupling method according to BIAcore standard protocols. All immobilization reagents were provided from the BIAcore coupling kit. Regeneration of receptor was performed with 30 μl of 10 mM NaOH, pH 11 at 100 μl/min. APN was immobilized at three different densities from approximately 75–800 resonance units (RU). One flowcell per chip served as a control surface containing non-toxin-binding APN treated with mild alkaline hydrolysis. A buffer flow rate of 30 μl/min was used for both association and dissociation. Toxins were injected at five different toxin concentrations (100, 200, 500, 750, 1000 nM) in random order. The toxin–receptor complex was allowed to dissociate for at least 5 min after 4 min of injection.

SPR data were analyzed with global fitting in BIAevaluation 3.0. For Cry1Ac binding to *M. sexta* APN, a complex two-binding site (heterogeneous ligand) model (A+B1+B2 ↔ AB1+AB2) was employed. Rationale for fitting to this model has been described [13]. Response curves generated by Cry1Ac binding to *L. dispar* APN were analyzed using the two-state (conformational change) model, as described [17]. Apparent rate constants have standard errors less than 10% of the values reported.

3. Results and discussion

3.1. Expression of mutant toxins and biological activities

The Cry1Ac α8 loop residues mutated in this study are shown in Fig. 1. Alanine substitution mutant toxins, N275A, F276A, D277A, S279A, F280A, R281A, S283A, R289A, S290A, and S293A produced stable protoxins and the yields were comparable to wild-type. After trypsin digestion, all mutant proteins produced stable toxin fragments like wild-type, as determined by SDS–PAGE and CD spectra analysis (data not shown). Biological activities of the mutant toxins against *L. dispar* and *M. sexta* larvae were determined. The mutant toxins, N275A, F276A, D277A, S279A, F280A, S283A, S290A, and S293A exhibited similar toxicity as Cry1Ac toxin to both insects. The LC_{50} values ranged from 2.5 to 5.7 ng/cm^2 to *M. sexta* larvae and 5.9–10.3 ng/cm^2 to *L. dispar* larvae. On the other hand, two arginine mutant toxins, R281A and R289A, showed great reductions in toxicity to both insects (Table 1). To *L. dispar* larvae, R281A and R289A toxins exhibited 100-fold and 49-fold reduced toxicity, respectively. To *M. sexta* larvae, R281A and R289A showed 48-fold and 30-fold reduced toxicity. These data strongly suggest that the arginine residues in this loop are important for toxicity.

3.2. BBMV-binding assays

BBMV competition binding assays were performed with

Table 1
Biological activity and affinity of Cry1Ac mutant toxins to *L. dispar* and *M. sexta*

Insect	Toxin	LC_{50} (ng/cm^2)[a]	Toxicity loss[b]	K_{com} (nM)[c]	K^b_{D1} (nM)	K^c_{D2} (nM)
L. dispar	Cry1Ac	7.5	1	2.3	–	210
	R281A	749	100	34.4	–	850
	R289A	365	49	65.3	–	525
M. sexta	Cry1Ac	5.3	1	5.3	100	77
	R281A	255	48	25.3	165	267
	R289A	157	30	54.5	94	123

[a]50% lethal concentration.
[b]LC_{50}mut/LC_{50}wt.
[c]BBMV-binding affinity.

iodine-labeled Cry1Ac toxin. To *L. dispar* BBMV, wild-type Cry1Ac binds with high affinity, yielding a K_{com} of 2.3 nM. The toxic mutant proteins (N275A, F276A, D277A, S279A, F280A, S283A, S290A, and S293A) bound to BBMV with affinities similar to Cry1Ac (data not shown). However, R281A and R289A mutant toxins bound to BBMV with lower affinities of 34.4 and 65.3 nM, respectively (Fig. 2). To *M. sexta* BBMV, wild-type Cry1Ac binds with a K_{com} of 5.3 nM. The toxic mutant proteins (N275A, F276A, D277A, S279A, F280A, S283A, S290A, and S293A) bound to BBMV like wild-type (data not shown), whereas R281A and R289A mutant toxins bound to BBMV with reduced affinities of 25.3 nM and 54.5 nM, respectively (Fig. 2). BBMV competition binding data are well correlated with toxicity data, in which less toxic proteins (R281A and R289A) bound to BBMVs with lower binding affinities. Dissociation binding assays were also performed to determine whether the loss of toxicity and competition binding were influenced by irreversible binding properties of the mutant toxins. No measurable differences between mutants and wild-type were observed in dissociation binding assays (data not shown). This indicated that the loss of BBMV affinity might be solely due to loss of reversible BBMV binding, presumably caused by a reduced affinity for membrane receptors.

3.3. Kinetic analysis of APN receptor binding by SPR

In order to further examine the molecular basis of the reduction in toxicity and BBMV binding of Cry1Ac mutant toxins, SPR-binding assays were performed with Cry1Ac receptor APN purified from *L. dispar* and *M. sexta*. Previously, much lower binding affinity has been reported for Cry1Ac toxin binding to purified APN using SPR compared to Cry1Ac toxin affinity for BBMV [15]. In contrast, a 210-kDa cadherin-like protein identified as a Cry1Ab receptor

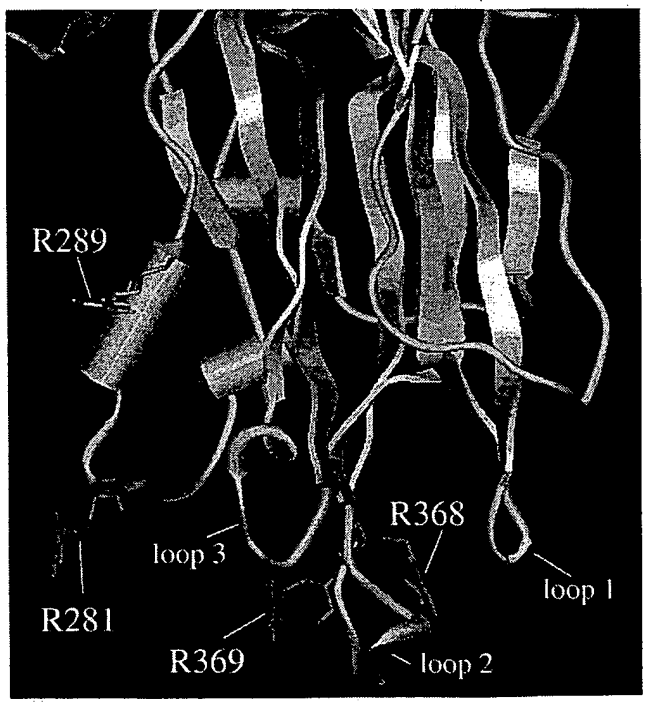

Fig. 1. Ribbon display of the Cry1Ac domain II predicted three-dimensional structure. R281, R289, R368 and R369 residues mutated in this study are shown in stick mode.

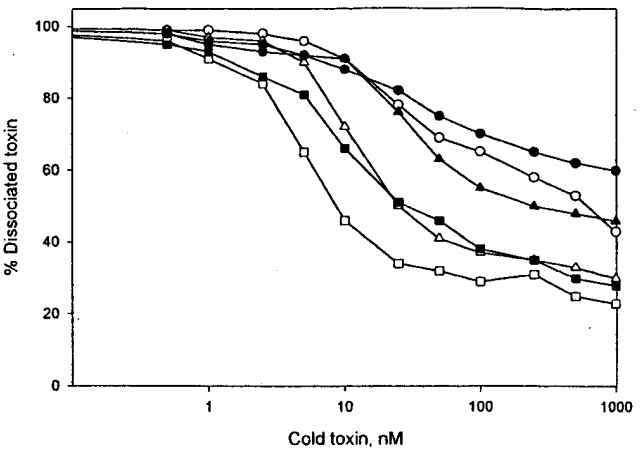

Fig. 2. Binding of Cry1Ac mutant toxins to *L. dispar* BBMV and *M. sexta* BBMV. ^{125}I-labeled Cry1Ac toxin (2 nM) was incubated with 10 μg of BBMV in the presence of increasing concentration of unlabeled Cry1Ac. Binding is expressed as a percentage of the amount bound upon incubation with labeled toxin alone. Open symbols, *M. sexta*; filled symbols, *L. dispar*. Cry1Ac toxin, squares; R289A toxin, triangles; and R281A toxin, circles.

in *M. sexta* showed binding affinities for Cry1Ab toxin similar to BBMV [12], although the value was not obtained by SPR. The question of how many receptors function in toxicity still remains to be answered. Fig. 3A shows the sensorgram of Cry1Ac and mutant toxins, R281A and R289A, binding to *M. sexta* APN. Both R281A and R289A displayed considerable reductions in binding to *M. sexta* APN compared to wild-type Cry1Ac. Fig. 3B shows the sensorgram of Cry1Ac and mutant toxins, R281A and R289A to *L. dispar* APN. The mutant toxins also showed weaker binding to *L. dispar* APN. The reduction in overall binding affinities for *M. sexta* APN appears to derive primarily from one of the two sets of rate constants (K_{D2}, data available on request), where R281A and R289A showed 3.5-fold and 1.6-fold losses in affinity, respectively (Table 1). These data suggest that the α8 residues important for binding and toxicity might be involved in binding to one of the *M. sexta* APN-binding sites. Alterations in both the rates of complex association (k_{a2}) and dissociation (k_{d2}) were obtained (data not shown). Similarly, the reduction in overall K_D for the mutant binding to *L. dispar* APN was due to alterations in k_{a2} and k_{d2} apparent rate constants. R281A and R289A showed 4-fold and 2.5-fold losses in affinity to *L. dispar* APN overall. The correlation of our mutants' biological activity and APN binding further supports a functional role for APN in Bt toxicity to lepidopterans.

In this study, novel α8 loop mutations in domain II of Cry1Ac were shown to affect binding to BBMV and purified APN receptor, which diminished insecticidal activity toward the lepidopteran pests *L. dispar* and *M. sexta*.

Although the α8 mutations in this study occur in domain II of Cry1Ac, recent SPR studies employing mutagenesis of domain III or domain swapping have shown that domain III is critical for Cry1Ac binding to purified APN from either *L. dispar* or *M. sexta* [16,17]. Further, the contact of Cry1Ac with *M. sexta* and *L. dispar* APN appears to be initiated by docking of an *N*-acetylgalactosamine moiety on APN in a Cry1Ac domain III cavity [17,20,21]. Despite the importance of domain III for APN binding, no direct correlation with toxicity has been observed for either *L. dispar* or *M. sexta*.

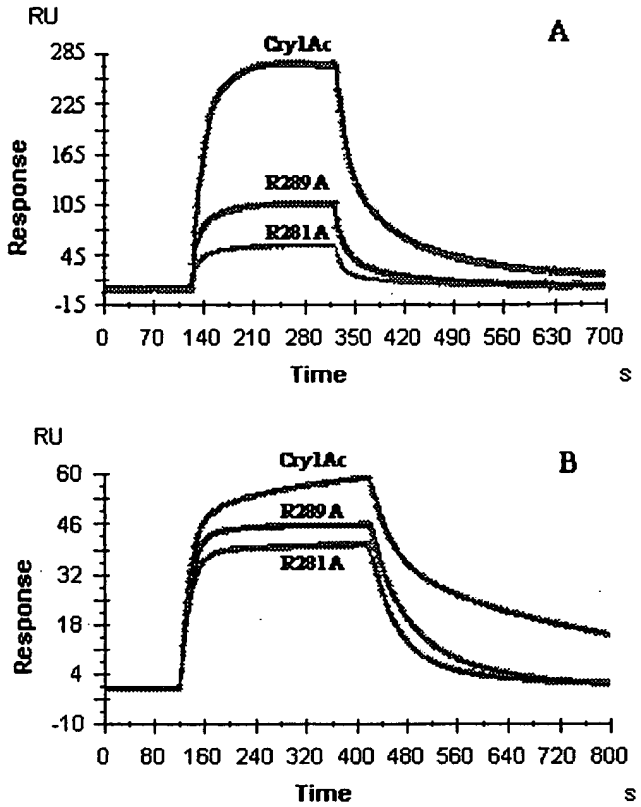

Fig. 3. Binding of Cry1Ac and mutant toxins to immobilized lepidopteran APNs. Toxins diluted in HBS buffer were injected over immobilized APN at 30 μl/min, 25°C. For dissociation, toxin flow was replaced by HBS buffer and recorded for at least 300 s. Experimental curves are gray and simulated fitted curves are overlaid in black. A: A representative binding curve overlay of Cry1Ac and mutant toxins (250 nM) injected over *M. sexta* APN (300 RUs) for 240 s. B: A representative binding curve overlay of Cry1Ac and mutant toxins (250 nM) injected over *L. dispar* APN (150 RUs) for 300 s.

Although domain III mutant toxins (Q509A, R511A, 509QNR-AAA511) eliminated APN binding, toxicity decreased no more than 2-fold, suggesting an alternative receptor for Cry1Ac is present in BBMV from both these insects. Therefore, analysis of toxin binding by domain III mutants to APN is insufficient for predicting toxicity [17,19,21]. On the other hand, R281A and R289A, as well as several other domain II loop mutations, not only affect APN binding but retain strong correlation between toxicity and binding affinity [17]. Despite this correlation, the loss in BBMV binding and toxicity to *L. dispar* and *M. sexta* by R281A and R289A mutant toxins does not appear to be accounted for entirely by loss of APN binding. Previously, ligand blotting experiments have been used to localize the receptor-binding domain using domain-switched hybrid toxins. These studies provided evidence that domain II of Cry1Ac might be involved in binding the 210-kDa *M. sexta* receptor [18]. It is likely that domain II mutations, such as R281A and R289A, affect binding to other functional receptors in addition to APN, allowing binding and toxicity to better correlate than domain III mutant toxins. Alternatively, domain II may play a greater role in toxicity beyond receptor recognition, such as facilitating a conformational change for membrane insertion.

Arginine substitutions similar to those in this report have been introduced in the α8 loop of Cry1Aa and the mutant toxins were tested to both insects. R281A mutant toxin was not stable by trypsin digestion. Only R286A mutant toxin greatly affected toxicity and BBMV binding to *L. dispar* (M.K. Lee and D. Dean, unpublished). From these studies, arginine residues in the α8 loop seemed to be involved in initial recognition of receptors for Cry1Aa as well. Another study with Cry1Ab and Cry1Ac double arginine mutant toxins (R368/R369) also supports that the positive charges are involved in the initial recognition to the receptor on BBMV [17,22]. Pertinent to this discussion is the observation that the pH of the alimentary canal of various lepidopteran species ranges from 9 to 11.5, depending on the position in the midgut [30]. Since the p*K* of the arginine guanidino group is above 12, this may represent an important long-range force involved in orienting the correct toxin-binding position on receptors with a presumably negative surface. Overall, positive charges may play a conserved role in Cry toxin affinity for receptors in lepidopteran midguts.

Acknowledgements: We would like to thank Dr. Douglas L. Dahlman and Dr. Gary Bernon for the generous supply of *M. sexta* and *L. dispar* eggs, respectively. This research was funded by a Grant from the National Institute of Health, Allergies and Infectious Diseases, RO1 AI29092-08.

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1997, p. 3978–3984
0099-2240/97/$04.00+0

Vol. 63, No. 10

Single-Site Mutations in the Conserved Alternating-Arginine Region Affect Ionic Channels Formed by CryIAa, a *Bacillus thuringiensis* Toxin†

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Received 26 March 1997/Accepted 17 July 1997

The role of the third domain of CryIAa, a *Bacillus thuringiensis* insecticidal toxin, in toxin-induced membrane permeabilization in a receptor-free environment was investigated. Planar lipid bilayer experiments were conducted with the parental toxin and five proteins obtained by site-directed mutagenesis in block 4, an arginine-rich, highly conserved region of the protein. Four mutants were constructed by replacing the first arginine in position 21 by a lysine (R521K), a glutamine (R521Q), a histidine (R521H), or a glutamic acid (R521E). A fifth mutant was obtained by replacing the fourth arginine by a lysine (R527K). Like CryIAa, the mutants formed cation-selective channels. A limited but significant reduction in channel conductance was observed for all mutants except R521H. The effect was more dramatic for the voltage dependence of the channels formed by R521K and R521Q, which was reversed compared to that of the parental toxin. This study provides the first direct evidence of a functional role for domain III in membrane permeabilization. Our results suggest that residues of the positive arginine face of block 4 interact with domain I, the putative pore-forming region of CryIAa.

The crystals produced by the Gram-positive bacterium *Bacillus thuringiensis* during sporulation contain a variety of proteins that are toxic to larvae from lepidopteran, dipteran, and coleopteran insects (14). At the molecular level, it is believed that pore-related increased permeabilization of the apical membrane of midgut target cells constitutes the decisive step which results in cellular ionic imbalance and ultimately cell death (10, 15, 16). CryIC toxin forms ion channels in live cells from the Sf9 (*Spodoptera frugiperda*) and the UCR-SE1a (*Spodoptera exigua*) cell lines (23, 25). This protein also partitions in receptor-free planar lipid bilayers (26). Several other Cry toxins form ion-selective pores in planar lipid bilayers: CryIAa (11), CryIAc (28, 29), CryIIIA (29), CryIIIB2 (30), and CryIIA (6). However, little is known about the pore formation process or about the biophysical properties of the channels, their regulation or their molecular architecture.

It has been proposed (5, 9) that activated *B. thuringiensis* toxins have two distinct functional domains: a toxicity region (the first half of the protein) and a binding region (its second half). Highly conserved amino acid tracts are found in the toxic region (blocks 1 and 2) and in the binding region (blocks 3, 4 and 5) (14). It is believed that these regions play an important role in the insecticidal activity of Cry toxins. Recently, structural data have been obtained by X-ray crystallography of CryIIIA, a coleopteran toxin (20), and CryIAa, a lepidopteran toxin (11). They show that the two toxins share a three-domain tertiary structure. Among the conserved blocks, block 2 is located mainly in domain I, which is thought to be involved in transmembrane channel formation. Block 2 includes α_7, the last helix of helix-rich domain I, and the flexible loop which links domain I to domain II. Two other conserved tracts, block 4 and block 5, are located in domain III, whose role is believed to be limited to the maintenance of the protein structural stability. In particular, the fifth conserved tract has been shown to be critical to the production of properly conformed CryIVA toxins but had no effect on their insecticidal activity (24). Block 4, which is part of β-sheet 17 of CryIAa, is of particular interest. This region of domain III, RYRVRIR (R, arginine; Y, tyrosine; V, valine and I, isoleucine) from position 521 to position 527, presents a strongly positively charged face and a highly hydrophobic face. It interacts intramolecularly with block 2 of domain I via a hydrogen bond between the side chain of the third arginine (position 525) and a carbonyl oxygen from the arginine in position 254 of block 2, with the latter being the first residue in the loop which links domain I and domain II (11).

Mutational studies were conducted on block 4 of CryIAa (3). Although mutations in the second and third arginine positions resulted in altered structure and expression of the protein, other mutations directed to the first or the fourth arginine provided products that were essentially similar to the wild-type toxin in structure, as assessed by trypsin susceptibility and circular dichroism spectra, and in binding to brush border membrane vesicles from silkworm (*Bombyx mori*) midguts. However, these mutants exhibited significant functional differences compared to CryIAa. In vivo toxicity to *B. mori* was reduced, and the mutants were substantially less effective in inhibiting the short-circuit currents recorded from voltage-clamped silkworm midguts (3). These results suggest that block 4 plays a role in postbinding events, particularly at the level of membrane permeabilization. Further work on these mutants has been conducted recently to determine their effect on *B. mori* brush border membrane vesicle permeability by using light-scattering detection of vesicle swelling (31). It supported

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† NRCC publication 49508.

the previous results of Chen et al. (3) and demonstrated that mutations in domain III do indeed affect CryIAa toxin function at the midgut membrane level.

In the present study we examined the role played by block 4 of domain III in CryIAa channel formation in receptor-free, artificial phospholipid membranes. By combining single-site mutagenesis in block 4, the conserved alternating-arginine segment of domain III, with functional studies using ion channel reconstitution into planar lipid bilayers, we demonstrated directly that domain III of CryIAa plays a role in membrane permeabilization induced by the toxin. More specifically, our results show that both the conductance and the voltage dependence of the toxin-mediated channels are sensitive to single-residue changes made in the conserved block 4 of domain III.

MATERIALS AND METHODS

Mutagenesis and toxin expression and preparation. Mutant genes (31) were expressed in *Escherichia coli* JM103 cells which were grown for 2 days. Inclusion bodies were prepared and solubilized in sodium carbonate buffer at pH 9.5. Toxins were obtained by trypsin digestion of protoxins at a final trypsin/protoxin ratio of 1:25 (wt/wt) for at least 4 h at 37°C. Small peptides were removed by column chromatography (fast protein liquid chromatography-Mono Q; Pharmacia Biotech, Piscataway, N.J.). Digested toxins were dialyzed overnight against sodium carbonate buffer and then examined by sodium dodecyl sulfate (10%)–polyacrylamide gel electrophoresis (17). For bilayer work, toxins were precipitated with 40% ammonium sulfate and resuspended in 150 mM KCl buffered with 25 mM Tris at pH 9.0 to prepare the toxin stock solution (0.8 to 1.5 mg of protein per ml). All chemicals were obtained from Sigma (St. Louis, Mo.), unless noted otherwise.

Planar lipid bilayer methods and data analysis. Planar lipid bilayers (26) were formed from a 7:2:1 lipid mixture (final concentration of 25 mg/ml in decane) of phosphatidylethanolamine, phosphatidylcholine, and cholesterol (Avanti Polar Lipids, Alabaster, Ala.). The bilayer was painted, using disposable glass rods made from prepulled, sealed-tip Pasteur pipettes, across a 250-μm orifice drilled in a Delrin cup (*cis* chamber) and pretreated with the above-described lipid mixture dissolved in chloroform. Membrane thinning was monitored by visual observation through a binocular dissection microscope and was assayed electrically by applying a triangular voltage to the membrane. Typical membrane capacitance values ranged between 150 and 250 pF. Channel activity following addition of trypsin-activated toxin or its mutants to the *cis* chamber at 5 to 20 μg/ml (85 to 340 nM) was monitored by step changes in the current recorded during holding test voltages across the planar lipid bilayer. Toxin incorporation was facilitated by stirring the buffer in the *cis* chamber with a magnetic stir bar and by applying a holding voltage of −80 mV. Temperature and pH were measured with a combined instrument (BAT-10/PHM-1) from Physitemp (Clifton, N.J.). All experiments were performed at room temperature (20 to 22°C) in buffer solutions containing either 150 or 450 mM KCl, 1 mM $CaCl_2$, and 10 mM Tris, pH 9.0. Single-channel currents were recorded with an Axopatch-1D patch-clamp amplifier (Axon Instruments, Foster City, Calif.). The 5-kHz low-pass-filtered currents were pulse-code modulated (Instrutech Corp., Great Neck, N.Y.) and stored on VHF magnetic tape. For analysis, data were played back through an analog eight-pole, low-pass Bessel filter (Frequency Devices, Haverhill, Mass.) set at 600 Hz and digitized at a 2.5-kHz sampling frequency (Labmaster TL-125; Axon Instruments). Data analysis was performed on a personal computer with pClamp or Axotape software (Axon Instruments). For each applied voltage, current amplitudes were measured on the recorded traces. For some voltages, current amplitude histograms were generated. Due to the multichannel nature of most records, no attempt was made to compare the kinetic properties of the single channels formed by the various proteins tested in this study, as multichannel activity rendered the interpretation of open-time and closed-time analyses quite complicated (21). However, a quantitative estimate of channel activity was obtained by measuring t_o, the sum of the total open times spent by the N identical channels at each open state level, over t_i, the total recording time interval. N, the number of active channels in the bilayer, was obtained from channel current amplitude histograms, and P_o, the estimated mean probability of a channel being open, was calculated by $P_o = (t_o/t_i)/N$.

Subconductance states were recognized as described before (26) by using the following identification criteria (7): (i) direct transitions from subconductance levels to main conductance levels were observed, (ii) subconductance states were never observed in the absence of the main conductance state, and (iii) the main conducting state did not result from the superposition of two or more independent channel openings.

Applied voltages are defined with respect to the *trans* chamber, which was held at virtual ground. Positive currents (i.e., currents flowing through the planar lipid bilayer from the *cis* chamber to the *trans* chamber) are shown as upward deflections. The direction of current flow corresponds to positive charge movement.

Conductance data are given as means ± standard errors of the means (SEMs). The mean conductance of each mutant main conducting state was compared to that of the parental toxin by using a two-tailed Mann-Whitney U test ($P < 0.01$).

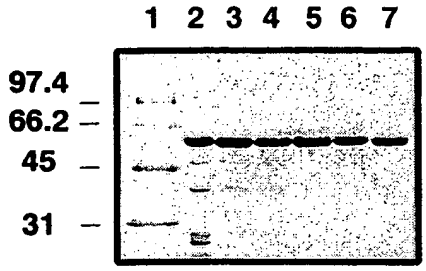


FIG. 1. Sodium dodecyl sulfate (10%)-polyacrylamide gel electrophoresis of trypsin-activated CryIAa and alternating-arginine mutant toxins. Lane 1, molecular size markers, with molecular masses (in kilodaltons) shown on the left; lane 2, R521E; lane 3, R521H; lane 4, R521K; lane 5, R521Q; lane 6, R527K; lane 7, CryIAa. The proteins were visualized by Coomassie brilliant blue staining.

RESULTS

CryIAa and its mutants form channels in planar lipid bilayers. Figure 1 shows that the wild-type protoxin as well as the arginine mutants were processed by trypsin to approximately 62-kDa toxic cores. The recombinant CryIAa used in this study formed channels that resembled closely those recorded under similar conditions after incorporation of wild-type CryIAa toxin purified by fast protein liquid chromatography (11). Similarly, the products obtained from single-site mutations of arginine in position 521 or 527 of the toxin partitioned in lipid membranes after a few minutes and at similar doses (85 to 340 nM). Well-resolved current jumps corresponding to the passage of ions through open channels were recorded as illustrated in Fig. 2 (traces).

Channel activity: multiple channels and substates. Usually, the number of active channels following the incorporation of CryIAa or its mutants into the planar lipid bilayer increased for a short period of time (5 to 10 min). It then remained stable for tens of minutes to hours, suggesting that progressive but rather limited incorporation took place in the planar lipid bilayer. Depending on the protein, more than one level of current jumps were observed, and for some of them, bursts of up to 10 different levels were regularly recorded. Qualitative differences in toxin channel activity were apparent (Fig. 2, traces). Compared to that of CryIAa, R521H partition into the lipid bilayer was more difficult: at an equivalent dose, it took longer for the onset of channel activity, and usually only one principal conducting level was seen, with long open times separated by long closed times (1 s or longer). This mutant did possess small subconducting states at positive voltages (Fig. 2, traces). The kinetics of R521K and R527K channels was generally similar to that of CryIAa, with multiple, stable openings of average duration (1 s or less). Subconducting states were apparent in R527K held at positive voltages. Similarly, the channels formed by R521E possessed several subconducting states when positively polarized. In contrast, R521Q subconducting states were observed only at negative voltages. This protein displayed a noisier activity under these conditions.

Site-directed mutations affect single-channel conductance, voltage dependence, and activity level of CryIAa channels. Single-channel conductances of CryIAa and its mutants were derived from the slopes of linear regression curves of single-channel current data recorded for several voltages applied across the lipid bilayer (I-V curves). For conductance determination, only the principal conducting states, i.e., the largest

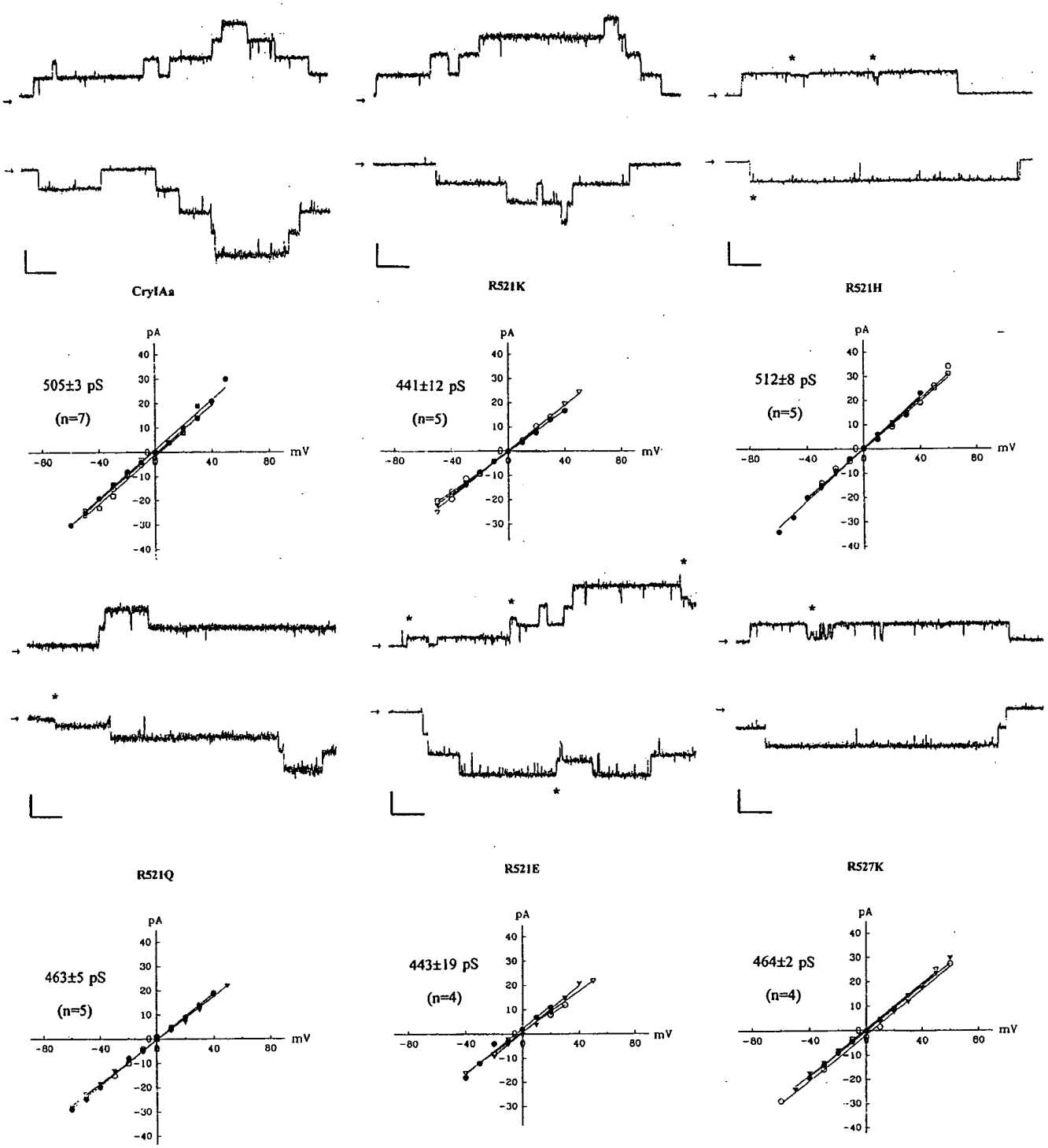


FIG. 2. The top parts of the panels show representative single-channel current traces recorded after partition of CryIAa and the five CryIAa mutants into planar lipid bilayers separating symmetrical KCl buffer solutions (150 mM in the *cis* chamber and 150 mM in the *trans* chamber) at pH 9.0. R521 mutants are shown in order of the charge shift sequence from positive to negative (R, K, H, Q, E). For the top traces, the applied voltage was +20 mV; for the bottom traces, the applied voltage was −20 mV. Arrows on the left of the traces indicate the closed state of the channels. Subconducting states are indicated by asterisks near the current traces. Scales: vertical bars, 10 pA; horizontal bars, 160 ms. The bottom parts of the panels show corresponding current-voltage relations (I-V curves). Data points from each individual experiment are represented by identical symbol types and were fitted by linear regression. Conductance values given in the upper left quadrants of the I-V curve graphs are means ± SEMs of the linear regression slopes.

TABLE 1. Single-channel properties and in vivo toxicities of wild-type CryIAa and five alternating-arginine mutants[a]

Activated toxin	Ion channel[b]			LD_{50} (ng/larva) (40–50 larvae)[c]
	Conductance (pS) (mean ± SEM)[d]	Mean P_o at:		
		−20 mV	+20 mV	
CryIAa	505.1 ± 2.5 (7)	0.37	0.25	4.5
R521H	512.2 ± 8.3 (5)	0.05	0.13	7.9
R521Q	463.4 ± 4.8[e] (5)	0.13	0.44	8.4
R521E	443.0 ± 19.2[e] (4)	0.62	0.38	8.4
R521K	441.2 ± 11.9[e] (5)	0.24	0.53	7.6
R527K	463.7 ± 2.2[e] (4)	0.04	0.06	7.7

[a] Conductance values of the R521 mutants are shown in decreasing order. This sequence differs from the charge shift sequence (R, K > H, Q > E) predicted for the 14-residue segment (PLSQRYRVRIRYAS) that includes the alternating-arginine region of block 4.

[b] With 5 to 20 μg of activated toxin per ml in the *cis* chamber and symmetrical (150 mM) KCl conditions.

[c] LD_{50}, 50% lethal dose. Results are reproduced from reference 31. Larvae were force fed 2 μl of activated toxin solution.

[d] The number of experiments for each toxin is shown in parentheses.

[e] Significantly different from CryIAa conductance by Mann-Whitney U test ($P < 0.01$).

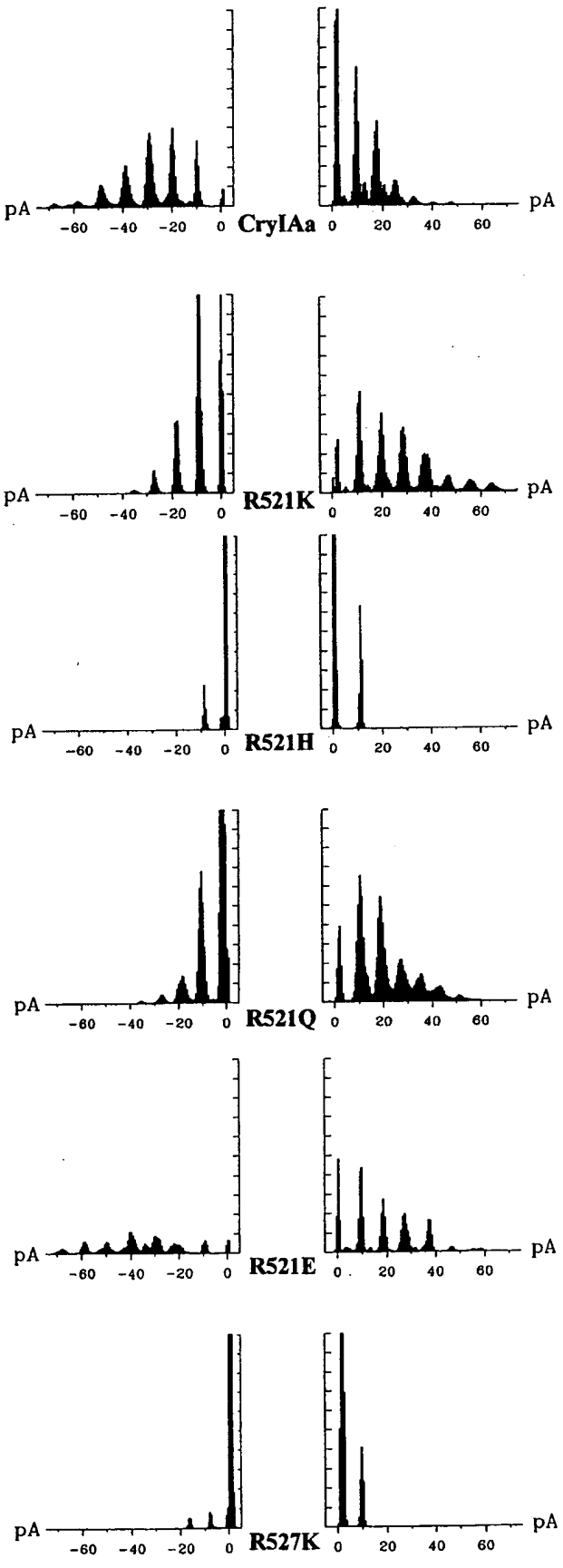
detectable current jumps for which direct transitions between the baseline and the conducting level, could be observed, were considered. Such I-V curves were obtained from current data recorded under symmetrical ionic conditions (Fig. 2) and under nonsymmetrical conditions (see Fig. 4). The I-V curves were rectilinear, which indicated that the channels passed current equally well in either direction. Conductance data obtained with 150 mM KCl in both the *cis* and the *trans* chambers are listed in Table 1. Replacement of R521 by a histidine did not affect channel conductance, but the channel conductances of the other mutants were significantly reduced, by 8.1 to 12.7%. With 450 mM KCl in the *cis* chamber and 150 mM KCl in the *trans* chamber, the conductance of CryIAa was 575 ± 53 pS ($n = 2$), the conductance of R521K was 540 ± 29 pS ($n = 3$), and the conductance of R527K was 654 ± 18 pS ($n = 2$).

All-point amplitude histograms were generated from 30-s, 600-Hz filtered current records for each protein held at −20 or +20 mV (Fig. 3). They provided a semianalytical tool (4, 21) which was used to compare CryIAa to its mutated counterparts. The number of distinguishable peaks observed in such histograms indicated the presence of multiple channels or subconductance states (or both). The area under each peak represents the occupancy probability of the channels at each specific current level. Overall channel dependence on voltage can be qualitatively assessed by means of these histograms, as voltage-independent channels exhibit similar current peak distributions, particularly at voltages of opposite polarity. Figure 3 shows that for most toxins, amplitude histograms generated at −20 mV were different from those generated at +20 mV, a clear indication that the channels formed by CryIAa and most of the mutated toxins were voltage dependent. Furthermore, the amplitude histograms of channels formed by CryIAa and the arginine mutants were markedly different. At +20 mV, CryIAa displayed several distinct, equidistant peaks. The amplitude of the leftmost peak, which corresponded to the closed state of all channels, was the largest. The next peaks were progressively smaller. At −20 mV, the amplitude histogram of CryIAa was different: the closed state was less frequent, as demonstrated by the small amplitude of the rightmost peak, and occupancy of the second and third levels was favored. Therefore, the mean probability of CryIAa channels being open was increased by negative voltage. A similar behavior was observed for R521E. In contrast, the situation was reversed for R521K and R521Q, for which the occupancy probability shifted to higher current levels with positive voltages. Finally, R521H and R527K amplitude histograms reflected the low level of activity of these two mutants. This may be explained by the fact that the mutated proteins were difficult to incorporate into planar lipid bilayers. Alternatively, it is also possible that these particular mutations affected the gating of the proteins in such a way that the channels they formed remained mostly closed. P_o, the mean probability of each mutant channel being open at two opposite voltages, was obtained from typical 30- to 90-s current records (Table 1). These data are consistent with the results of the amplitude histogram analysis described above. They show that in contrast to CryIAa and R521E, R521K and R521Q were activated by positive voltages.

Like those of CryIAa, the channels formed by the mutants are cation selective. Ionic selectivity was tested under nonsymmetrical ionic conditions (450 mM KCl in the *cis* chamber and 150 mM KCl in the *trans* chamber). The current-voltage relations of CryIAa and its mutants were determined as described above. They shifted to the left, with zero-current voltages, i.e., reversal potential becoming more negative by approximately 25 mV for CryIAa, 15 mV for R521K, and 18 mV for R527K, as illustrated in Fig. 4. The other mutants behaved the same way (not shown). This shift was consistent with a Nernst equilibrium potential of −27.7 mV calculated for monovalent cations under these conditions, demonstrating the selectivity of the channels for cations. It should be noted that the apparent reduction of selectivity for cations, compared to that of CryIAa, was observed in a small set of experiments. Determination of whether it reflects a true effect of mutation needs further investigation.

DISCUSSION

In this study, the lipid bilayer technique was used to compare the in vitro ion transport properties of wild-type CryIAa toxin to those of five proteins obtained by site-directed mutagenesis in the conserved alternating-arginine region of CryIAa. Two important biophysical properties of the channels were significantly altered by mutations in block 4 of the third domain of CryIAa: the conductance of most mutants was reduced and the voltage dependence of two of them, R521K and R521Q, was reversed, compared to those of the wild-type toxin. While this effect of mutation on conductance was not dramatic, it was statistically significant. In terms of residue substitution in position 521 of the protein, the order of conductances (from the largest to the smallest) was H, Q, E, K. This order did not follow that of a progressive charge shift from positive to negative predicted for the residue sequence that maps CryIAa conserved block 4. Interestingly, R521K, the most conservative mutation, and R521E, the least conservative mutation, affected similarly the conductance of the channel, while a histidine substitution had no effect. These results suggest that local charges in the alternating-arginine region are at best a minor determinant of CryIAa conductance. The second major effect of arginine mutation, i.e., reversal of the channel voltage dependence, was clearly apparent in the all-point amplitude histograms constructed for CryIAa and its alternating-arginine mutants and was confirmed by the results of P_o analysis. Therefore, while the voltage dependence of CryIAa channels was influenced by certain mutations in block 4, it did not appear to be regulated by the charge of the residue in position 521. Other factors, like residue size, side chain orientation and intra- or intermolecular interactions, may also play a critical role. It is



clear that further work is needed to clarify the complex nature of conductance and voltage regulation of Cry toxin channels.

Chen et al. (3) reported that arginine mutations in the fourth conserved block of CryIAa did not affect binding to *B. mori* brush border membrane vesicles. Compared to CryIAa, the mutants were approximately four times less active against second-instar silkworm larvae, as determined by diet surface contamination bioassays, and were less efficient in inhibiting the voltage clamp short-circuit current related to active transport across fifth-instar larvae midguts. Biological activity of the same mutated products as those used in the present investigation was tested with *B. mori* larvae by using a force-feeding bioassay (18). The results, which were reported elsewhere (31), are reproduced in Table 1 for convenient comparison with electrophysiological data. They show that compared to that of CryIAa, the toxicity of the mutants, i.e., the inverse of the 50% lethal dose, was reduced by 41 to 46%. In the same work (31), it was shown, using a light-scattering permeability assay on brush border membrane vesicles, that R521H, R521K, and R521Q exhibited the same membrane permeabilization capability as CryIAa, while R521E, R523K, R527H, and R527K were less active in the vesicle permeabilization assay. Our conductance data for R521E, R521H, and R527K are consistent with the light-scattering results. However, the mutants with lysine and glutamine in position 521 behaved differently in the two assays. The reasons for these discrepancies are not apparent, but they may be dependent on experimental conditions. It should be noted that both Chen et al. (3) and Wolfersberger et al. (31) conducted their experiments on biological preparations with functional CryIAa receptors. The results suggested that a change in ion transport properties of the mutated toxins took place, and it was concluded that mutations in the alternating-arginine region of CryIAa affected mainly the postbinding events related to toxicity. However, none of these studies could conclusively attribute the effect to altered pore function. In midgut voltage clamp experiments it is difficult to rule out metabolic pathways or paracellular permeabilities. Data from brush border membrane vesicle experiments report only the effects of toxins on membranes extracted from the luminal side of the midgut epithelium cell layer and in the absence of metabolic regulation. Furthermore, information obtained from these two experimental models is restricted to overall macroscopic ion transport, i.e., to the current whose total amplitude corresponds to the product of single-channel current amplitude, total number of channels present in the membrane, and probability of each channel being open (13).

Many of these interpretative difficulties can be reduced with the lipid bilayer approach, which provides direct biophysical information on single-channel properties of pore-forming proteins. Conductance, ionic selectivity, and, in the simplest cases, kinetic behavior can be ascribed precisely to the permeating pathway. On the other hand, the lipid bilayer technique also has its limitations: the artificial membrane may poorly mimic the actual membrane composition and environment in the gut, and of course there is no receptor and no metabolic regulation. Nevertheless, the present study demonstrates clearly that single-site mutations in block 4 of CryIAa resulted in altered

FIG. 3. All-point current amplitude histograms for 30-s recordings at −20 mV (left) and at +20 mV (right). R521 mutants are shown in order of the charge shift sequence from positive to negative (R, K, H, Q, E). Counts were distributed in 120 bins. They are shown on the vertical axes (4,000 counts/tick). Horizontal axes represent the current amplitude (pA). The rightmost peaks in the histograms on the left and the leftmost peaks in the histograms on the right correspond to the closed state of the channels at holding voltages.

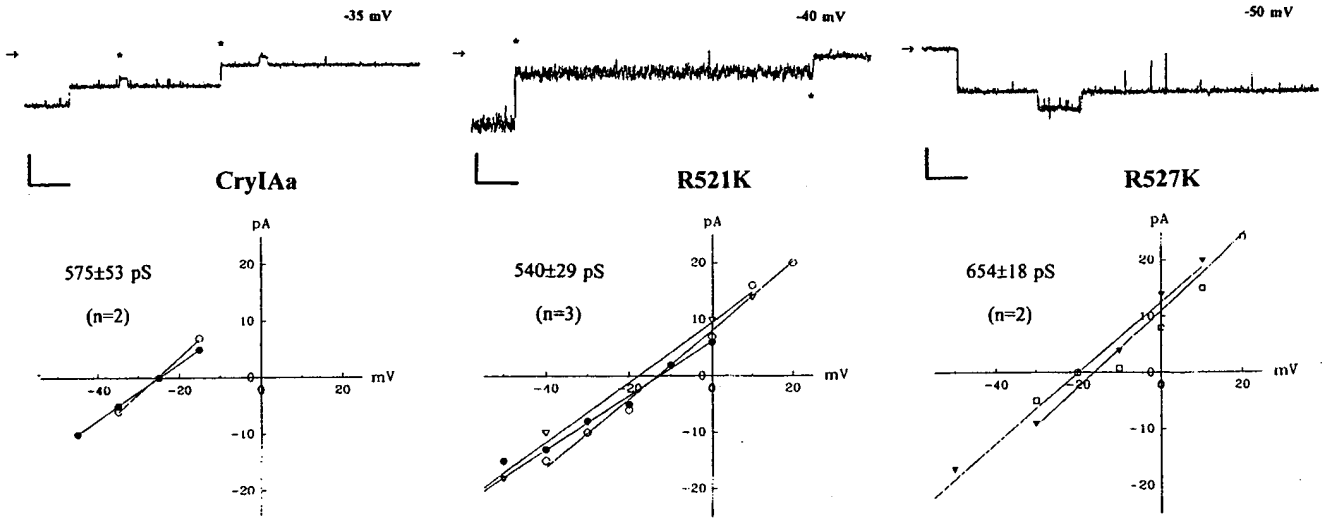


FIG. 4. The top parts of the panels show representative single-channel current traces recorded after partition of CryIAa and two R-to-K mutants into planar lipid bilayers separating nonsymmetrical KCl buffer solutions (450 mM in the *cis* chamber and 150 mM in the *trans* chamber) at pH 9.0. Applied voltages are indicated next to the traces. Arrows on the left of the traces indicate the closed state of the channels. Subconducting states are indicated by asterisks near the current traces. Scales: vertical bars, 10 pA; horizontal bars, 160 ms. The bottom parts of the panels show corresponding current-voltage relations. Data points from each individual experiment are represented by identical symbol types and were fitted by linear regression. Conductance values given in the upper left quadrants of the I-V curve graphs are means ± SEMs of the linear regression slopes. The direction and the magnitude of the reversal potential shifts (horizontal axis intercept) observed on the I-V curves demonstrate that CryIAa (−25.0 ± 1.1 mV), R521K (−14.7 ± 2.2 mV), and R527K (−18.2 ± 1.9 mV) are selective to cations.

functional properties of the protein. While all mutants retained the ability to partition into phospholipid membranes, R521H was significantly less effective in doing so, as only one channel opening level was usually observed. Furthermore, other crucial functions, such as conductance and voltage dependence, were affected by mutation. Considering that domain I of CryIAa, which is totally α-helical (11), is a leading candidate for pore formation, this study suggests that there are long-range interactions between domain I and the conserved alternating-arginine region of domain III and that these interactions do not depend solely on the charges of the mutated residues.

It has been proposed that Cry toxin insertion into membranes would be the result of "penknife"- or "umbrella"-like conformational changes triggered by binding of the toxin to its receptor (15). In the first model, the hairpin made of helices α_5 and α_6 flips into the cell membrane, and the rest of the molecule remains in the aqueous phase in a relatively unchanged conformation. In the second model, helices α_4 and α_5 enter the bilayer as a helical hairpin, and the other five helices of domain I flatten out on the membrane surface. An interesting feature of the umbrella model is that the region of the toxin that includes the alternating arginine segment moves closer to the area which could form the mouth of the putative channel formed by oligomeric association of other similarly unfolded toxins. Based on the data presented in this study and on the results of experiments conducted with disulfide-bridge-engineered Cry toxins (27, 32), it is tempting to propose that the alternating arginine region does indeed become part of, or interacts with, the mouth of the channel. Mutations in this region may therefore alter the channel mouth environment, which would result in reduced ion transport across the membrane and diminished biological activity.

In summary, this study demonstrates directly, and for the first time, that domain III, a part of the protein which has already been shown to play a role in binding and specificity determination (1, 2, 8, 19, 22), plays an important role in membrane permeabilization. More specifically, CryIAa toxin channel conductance and voltage dependence were significantly influenced by the alternating arginine segment of domain III. Furthermore, if the toxin pore is indeed formed by domain I, as has been proposed (11, 20), this study would also provide new direct evidence of a functional interaction between domain I and domain III in Cry toxins. Such an interaction between the N- and C-terminal regions of CryIAb has been implied at the level of toxin specificity (12). By providing new information on structure-function relations in CryIAa, this study will help to further our understanding of the molecular properties and the mode of action of *B. thuringiensis* toxins.

ACKNOWLEDGMENTS

This work was supported in part by grant OGP0171373 from the Natural Sciences and Engineering Research Council of Canada to R.L. and J.L.S. and by grant R01 AI29092 from the National Institutes of Health to D.H.D.

We thank A. Mazza, Biotechnology Research Institute, National Research Council of Canada, for expert technical assistance; P. Brink, State University of New York, Stony Brook, for stimulating suggestions on multiple-channel analysis; and P. Grochulski and L. Masson, Biotechnology Research Institute, National Research Council of Canada, for many helpful observations and valuable discussions.

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2002, p. 194–200
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.1.194–200.2002

Vol. 68, No. 1

Mutagenic Analysis of a Conserved Region of Domain III in the Cry1Ac Toxin of *Bacillus thuringiensis*

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Received 10 April 2001/Accepted 30 October 2001

We used site-directed mutagenesis to probe the function of four alternating arginines located at amino acid positions 525, 527, 529, and 531 in a highly conserved region of domain III in the Cry1Ac toxin of *Bacillus thuringiensis*. We created 10 mutants: eight single mutants, with each arginine replaced by either glycine (G) or aspartic acid (D), and two double mutants (R525G/R527G and R529G/R531G). In lawn assays of the 10 mutants with a cultured *Choristoneura fumiferana* insect cell line (Cf1), replacement of a single arginine by either glycine or aspartic acid at position 525 or 529 decreased toxicity 4- to 12-fold relative to native Cry1Ac toxin, whereas replacement at position 527 or 531 decreased toxicity only 3-fold. The reduction in toxicity seen with double mutants was 8-fold for R525G/R527G and 25-fold for R529G/R531G. Five of the mutants (R525G, R525D, R527G, R529D, and R525G/R527G) were tested in bioassays with *Plutella xylostella* larvae and ion channel formation in planar lipid bilayers. In the bioassays, R525D, R529D, and R525G/R527G showed reduced toxicity. In planar lipid bilayers, the conductance and the selectivity of the mutants were similar to those of native Cry1Ac. Toxins with alteration at position 527 or 529 tended to remain in their subconducting states rather than the maximally conducting state. Our results suggest that the primary role of this conserved region is to maintain both the structural integrity of the native toxin and the full functionality of the formed membrane pore.

Bacillus thuringiensis is a gram-positive bacterium that produces one or more insecticidal crystal (Cry) proteins deposited in the form of an intracellular parasporal crystal during sporulation (20). Shortly after ingestion by a susceptible insect, most Cry proteins dissolve in the insect midgut, are activated by midgut proteases, and bind to a specific midgut epithelial cell receptor. After binding to specific docking proteins on the microvillous surface of susceptible epithelial cells in the larval midgut (12, 27), the toxin undergoes a conformational change in which the helix-rich domain I (DI) separates from the other two domains (23). A hairpin, composed of helices α4 and α5, subsequently inserts into the membrane with the other five helices spreading, in an umbrella-like fashion, over the membrane surface (8), with α4 lining the lumen of the pore to create a functional ion channel (19). The toxin-exposed midgut epithelial cells eventually die by a colloid-osmotic lysis mechanism (14).

The atomic structures of two activated Cry proteins, Cry1Aa and Cry3A, have been reported (11, 16). The two proteins have similar three-domain structures. DI is directly involved in ion channel formation (8, 23, 28). The second domain, DII, is involved in binding (9, 17, 21), whereas DIII is thought to play both a structural role and a toxin recognition role (2, 5, 15, 17).

A multiple sequence alignment of *cry* genes shows five conserved blocks (13), with the fourth conserved block (in DIII) consisting of an intriguing series of four alternating arginines in a conserved β17 strand. In an earlier study of this region in Cry1Aa (4), mutations of the second and third arginines in the series resulted in mutants that were either poorly expressed (second arginine) or not expressed at all (third arginine). The second arginine of Cry1Aa is involved in a complex network of hydrogen bonding to adjacent β-strands, whereas the third arginine is in a salt-bridge triad with two other residues in addition to hydrogen bonding to other residues. Thus, the results suggest that the second and third arginines are important for structural stability (11). Mutations in the first and fourth arginine resulted in mutant toxins with wild-type receptor binding affinities but slightly decreased toxicity to larvae of *Bombyx mori*, suggesting that a postbinding event was affected (4, 29). Voltage clamping of insect midguts and brush border membrane vesicle permeabilization studies as well as planar lipid bilayer studies of the two arginine mutants demonstrated a possible role in the ion channel function of the toxin (4, 24, 29).

We chose Cry1Ac as a target for mutagenesis in the present study because unlike Cry1Aa (17), Cry1Ac is extremely lethal to cultured *Choristoneura fumiferana* (Cf1) insect cells (10, 18). Therefore, toxicity levels can be determined even if mutant toxin production is severely inhibited. Further, because Cry1Ac and Cry1Aa have 76% identity between amino acids 29 and 630, we could use the known structure of Cry1Aa as a template to produce a structural model of Cry1Ac with the comparative protein modeling server, Swissmodel (http://www.expasy.ch/swissmod/SWISS-MODEL.html).

The objective of the present study was to elucidate the func-

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tion of the four positively charged arginines in the conserved block 4 region of Cry1Ac at amino acid positions 525, 527, 529, and 531 in ion channel formation using planar lipid bilayers. Cultured insect cell lawn assays and larval bioassays were designed to determine if toxicity was severely affected in these mutants. To do this, we created eight single mutants and two double mutants of Cry1Ac with one or two arginines to either eliminate the positive charge (R-to-G replacement) or convert it to a negative charge (R-to-D replacement). Previous work with Cry1Aa examined only the first and last arginines, since mutations of the two middle arginines produced protoxins with altered solubility and proteolytic stability (4). The cultured insect cell lawn assay eliminates these problems by using only purified activated toxins, therefore bypassing in vivo solubilization and midgut protease activation. A subset of five mutants from those tested in the insect cell lawn bioassay were futher tested for toxicity in bioassays with *Plutella xylostella* larvae and for ion channel formation.

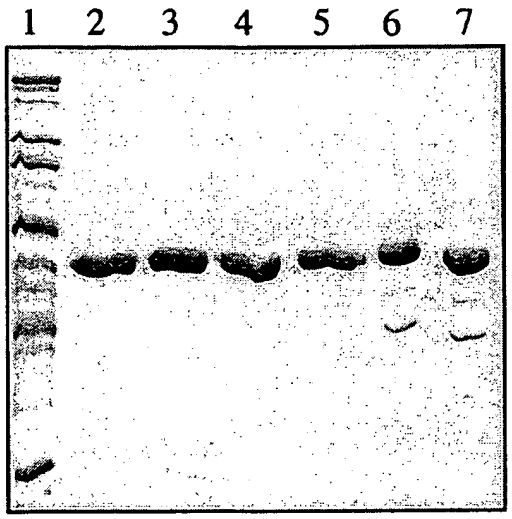

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel of purified, trypsin-activated Cry toxins. Lane 1, molecular weight markers; lane 2, Cry1Ac; lane 3, R525D; lane 4, R525G; lane 5, R527G; lane 6, R525G/R527G; lane 7, R529D. Approximately 2 μg of toxin was loaded per lane.

MATERIALS AND METHODS

Bacterial strains and plasmids. The double-stranded DNA plasmid pMP37 containing the *Bacillus thuringiensis* NRD-12 *cry1Ac* gene (GenBank accession no. U43606) (18) was used as a template for oligonucleotide-directed mutagenesis using the Transformer mutagenesis kit from Clontech (Palo Alto, Calif.). Mutant clones were sequenced using an automated fluorescent sequencer from Applied Biosystems, model 370A. Oligonucleotides were synthesized using an Applied Biosystems oligonucleotide synthesizer, model 394/4.

Toxin activation and purification. Purification of inclusion bodies expressed in *Escherichia coli* was done as previously described (18). Isolated inclusion bodies were solubilized and activated by incubating them at 28°C for 4 h in 0.04 M carbonate buffer, pH 10, with 1% trypsin (wt/vol), after which insoluble material was removed by centrifugation for 1 h at 200,000 × *g*. Activated toxins were purified by ion exchange fast-protein liquid chromatography by injecting the supernatant onto a Q-Sepharose column and subsequently eluting the bound toxin with a 0 to 500 mM NaCl gradient (18). A sample of the peak containing the purified toxin was stored at 4°C. For bilayer work, toxin stock solutions were prepared by resuspending water-precipitated toxin in 150 mM KCl buffered with 25 mM Tris-(hydroxymethyl)-aminomethane (Tris) at pH 9.0 to approximately 0.8 to 1.5 mg/ml protein). Samples were also resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer to assess toxin integrity and purity on an acrylamide gel (Fig. 1). The concentration of purified toxins were determined by the method of Bradford (3) using bovine serum albumin as a standard.

Insect cell lawn assays. Cf1 cells from trypsinized larval tissue of *C. fumiferana* (spruce budworm) were cultured in Grace's medium containing 0.25% tryptose and 10% fetal bovine serum. Cells were grown in spinner flasks at 28°C to a concentration of 1×10^6 to 2×10^6 cells/ml. In vitro toxicity assays were done on a lawn of agarose-suspended Cf1 cells (10). Activated toxins were diluted serially by half at each step, and 1 μl from each dilution was spotted onto a lawn of insect cells. After incubation at room temperature for 1 h, the plate was stained for 10 min with 20 ml of 0.2% (wt/vol) trypan blue in morpholinepropanesulfonic acid (pH 10) and destained for 60 min in 0.1 M morpholinepropanesulfonic acid (pH 10) followed by several hours in 1.34% (wt/vol) KCl. We measured the threshold concentration, which is the lowest concentration of activated toxin in the dilution series producing a visible trypan blue-stained spot on the immobilized cell layer. The data represent the average toxicity value between three to nine replicates for each toxin examined.

Larval bioassays. Larvae from the susceptible LAB-P strain of *Plutella xylostella* (diamondback moth) from Hawaii were used in leaf residue bioassays (25). Groups of 9 to 11 larvae ate cabbage leaf disks that had been dipped in distilled water dilutions of the protoxin form of each of five mutant Cry toxins. The concentration tested was 10 mg of protoxin per liter. In all tests, a surfactant (0.2% Triton AG98; Rohm and Haas) was added. Mortality was recorded after 5 days. Each test was replicated twice. The mean mortality of control larvae that ate leaf disks dipped in distilled water with surfactant only was 4% (range, 0 to 10%). Previous results in similar tests showed that 10 mg of native Cry1Ac protoxin per liter killed 94% of LAB-P larvae (26).

Planar lipid bilayers. Planar lipid bilayers (22) were formed from a 7:2:1 lipid mixture (25-mg/ml final concentration in decane) of phosphatidylethanolamine, phosphatidylcholine, and cholesterol (Avanti Polar Lipids, Alabaster, Ala.). The bilayer was painted, using disposable glass rods made from prepulled, sealed-tip Pasteur pipettes, across a 250-μm hole drilled in a Delrin cup (*cis* chamber) and pretreated with the above-described lipid mixture dissolved in chloroform. Membrane thinning was monitored by visual observation through a binocular dissection microscope and was assayed electrically. Typical membrane capacitance values ranged between 150 and 250 pF. Channel activity following addition of 5 to 10 μg (85 to 170 nM) of trypsin-activated toxin or its mutants/ml to the *cis* chamber was monitored by step changes in the current recorded while holding test voltages across the planar lipid bilayer. Toxin incorporation was helped by stirring the buffer in the *cis* chamber using a magnetic stir bar and by applying a holding voltage of −80 mV. All experiments were performed at room temperature (20 to 22°C) in buffer solutions containing either 150 or 450 mM KCl, 1 mM $CaCl_2$, and 10 mM Tris, pH 9.0. Single-channel currents were recorded with an Axopatch-1D patch-clamp amplifier (Axon Instruments, Foster City, Calif.). The 5-kHz low-pass-filtered currents were pulse-code modulated (Instrutech Corp., Great Neck, N.Y.) and stored on VHF magnetic tape. For analysis, data were played back through an analog eight-pole, low-pass Bessel filter (Frequency Devices, Haverhill, Mass.) set at 600 Hz and digitized at a 2.5-kHz sampling frequency (Labmaster TL-125; Axon Instruments). Data analysis was performed on a personal computer using pClamp or Axotape software (Axon Instruments). For each applied voltage, current amplitudes were measured on the recorded traces.

Subconductance states were recognized as described previously (22) using the following criteria (6): (i) direct transitions from subconductance levels to main conductance levels were observed; (ii) subconductance states were never observed in the absence of the main conductance state; and (iii) the main conducting state did not result from the superposition of two or more independent channel openings. Conductance data are given as means ± standard errors of the means.

Applied voltages are defined with respect to the *trans* chamber, which was held at virtual ground. Positive currents (i.e., currents flowing through the planar lipid bilayer from the *cis* chamber to the *trans* chamber) are shown as upward deflections. The direction of current flow corresponds to positive charge movement.

RESULTS

Cry1Ac mutagenesis. To assess the effect the alternating arginine region has on toxicity, individual arginines in Cry1Ac were replaced with a polar (glycine) or negatively charged (aspartic acid) amino acid (see Tables 1 and 2). In two clones,

TABLE 1. Bioassay of Cry1Ac arginine replacement mutants on *P. xylostella* larvae and cultured Cf1 cells

Toxin	a.a. sequence (525–532)[a]	DNA sequence (bases 1573–1596)[b]	Threshold toxicity [ng/μl (SE)]	Relative toxicity[c]	% Mortality (SE)[d]
Cry1Ac	RYRVRVRY	aga tat cga gtt cgt gta cgg tat	0.10 (0.03)	1.00	94[e]
R525G	G	g-- --- --- --- --- --- --- ---	0.54 (0.11)	0.18	80 (10)
R525D	D	gac --- --- --- --- --- --- ---	1.19 (0.40)	0.08	70 (0)
R527G	G	--- --- g-- --- --- --- --- ---	0.19 (0.10)	0.53	100 (0)
R527D	D	--- --- gac --- --- --- --- ---	0.20 (0.10)	0.50	ND[f]
R529G	G	--- --- --- --- g-- --- --- ---	0.39 (0.07)	0.26	ND
R529D	D	--- --- --- --- gac --- --- ---	0.57 (0.16)	0.18	65 (5)
R531G	G	--- --- --- --- --- --- g-- ---	0.14 (0.01)	0.71	ND
R531D	D	--- --- --- --- --- --- gac ---	0.30 (0.01)	0.33	ND
R525G/R527G	G G	g-- --- g-- --- --- --- --- ---	0.78 (0.16)	0.13	45 (5)
R529G/R531G	G G	--- --- --- --- g-- --- g-- ---	2.47 (0.16)	0.04	ND

[a] a.a., amino acid.
[b] Hyphens represent wild-type *cry1Ac* DNA sequence.
[c] Relative toxicity was calculated as the threshold concentration for native Cry1Ac divided by the threshold concentration for each toxin.
[d] Each mortality estimate is based on two replicates with 9 to 11 *P. xylostella* larvae tested per replicate. Mortality was not adjusted for control mortality, which ranged from 0 to 10% (mean, 4%). SE, standard error.
[e] Average Cry1Ac percent mortality as reported in prior assays (27).
[f] ND, Not done.

a pair of consecutive arginines was replaced with glycine to determine whether a greater decrease in the overall positive charge of this region would influence toxicity (Table 1).

Insect cell lawn assays. Changes in the first (R525) or second (R527) arginine position resulted in either the largest decreases in toxicity by a single mutation (5.4- to 11.9-fold) (R525) or no overt decrease (R527) compared to the wild-type Cry1Ac threshold value (Table 1). Changes in the third (R529) position resulted in a 3.9- to 5.7-fold decrease in toxicity towards Cf1 cells, whereas mutations in the fourth arginine (R531) caused little (3-fold) (R531D) or no (R531G) decreases in toxicity. In general, mutation of the positively charged arginine to negatively charged aspartic acid had a greater effect on toxicity than the change to a neutral glycine. The creation of a double mutant by replacement of arginine with glycine in the first two arginine positions (R525G/R527G) resulted in decreased threshold toxicity levels, which appeared to be additive when compared to effects of the two individual positions. This additive effect suggests that the overall positive charge in the first part of the arginine region is only of moderate importance. In contrast, neutralizing the charge in the latter two positions produced a clone with the largest decrease in toxicity (more than 24-fold), which is much greater than the sum of the two individual mutations alone.

Larval bioassays. Five mutant toxins (R525D, R525G, R527G, R529D, and R525G/R527G) that were 1.9- to 11-fold less toxic than Cry1Ac in lawn assays were tested further in larval bioassays as well as planar lipid bilayers (Table 1). Interestingly, this group included toxins from the two internal positions that previous Cry1Aa studies in the β17 strand region showed to have altered solubility or proteolytic stability (4). Since activated Cry toxins possess numerous internal trypsin cleavage sites, a good method of assessing deviations from wild-type toxin conformations is to monitor mutant protoxin solubility and proteolytic activation. The purified protoxins isolated from these mutants appeared similar to wild-type Cry1Ac in that they were highly soluble in alkaline buffer and showed normal activation by trypsin (Fig. 1). Although R527G had near-wild-type toxicity as did R531G in lawn assays, it was included for comparison purposes with the double mutant R525G/R527G. The double mutant R529G/R531G could not be included in further tests because its expression in *E. coli* was poor and sufficient amounts of the purified toxin could not be obtained.

Mean mortality (and standard error) caused by the mutant toxins in the larval bioassay was 100% (0) for R527G, 80% (10) for R525G, 70% (0) for R525D, 65% (5) for R529D, and 45% (5) for the double mutant R525G/R527G. In previously reported results with similar larval bioassays, mean mortality caused by native Cry1Ac was 94% (26). The three mutants that had the lowest toxicity in the larval bioassay (R525D, R529D, and R525G/R527G) also had greatly reduced toxicity in the lawn assay (relative toxicities, 0.08 to 0.18; see Table 1). However, R527G killed 100% in the larval bioassay, but its relative toxicity was 0.53 in the lawn assay. Similarly, R525G killed 80% in the larval bioassay, but its relative toxicity was only 0.18 in

TABLE 2. Conserved block 4 arginine region

Toxin[a]	Protein sequence[b]
Cry1Aa	R Y R V R I R Y A S
Cry1Ac	R Y R V R V R Y A S
Cry1B	R Y R I G F R Y A S
Cry1C	R Y R L R F R Y A S
Cry1D	S Y Y I R F R Y A S
Cry1E	R Y R L R F R Y A S
Cry1F	R Y R A R I R Y A S
Cry1G	Q Y R I R V R Y A S
Cry1H	Q Y R L R V R F A S
Cry1J	R Y R V R I R Y A S
Cry2Aa	S Y N L Y L R V S S
Cry2Ab	S Y N L Y L R V S S
Cry3A	K Y R A R I H Y A S
Cry3B	R Y R V R I R Y A S
Cry3C	K Y R V R V R Y A T
Cry3D	T Y K I R I R Y A S
Cry4A	S Y F I R I R Y A S
Cry4B	S Y G L R I R Y A A
Cry5A	E Y Q I R C R Y A S
Cry5B	Q Y T I R I R Y A S

[a] Sequences were obtained from the following Genbank nucleotide accession numbers (in order): U43605, U43606, X06711, X07518, X54160, X56144, M63897, X58534, Z37527, L32019, M31738, X55416, M22472, A07234, M64478, X59717, Y00423, X07423, L07025, L07027.
[b] Shaded letters represent deviations from the alternating arginine sequence found in conserved block 4 (see reference 13).

the lawn assay. Differences between the two assays could have been caused by interspecific differences (*C. fumerifana* in the lawn assay versus *P. xylostella* in the larval bioassay), differences in the assay technique (cultured cells versus live larvae), or both.

Planar lipid bilayers. The wild-type Cry1Ac toxin and the products obtained from mutation of arginine in position 525, 527, or 529 of the toxin partitioned in phospholipid membranes after a few minutes and at similar concentrations (85 to 170 nM). Well-resolved current jumps were observed, as illustrated in Fig. 2 (upper panels). The number of active channels following protein incorporation into the planar lipid bilayer was variable. In some experiments, it increased from one to four or five during the first 5 to 10 min and remained stable thereafter for tens of minutes to hours.

Single-channel conductances of Cry1Ac and its mutants were derived from the slopes of linear regression curves (I-V curves) of channel current data recorded for several voltages applied across the lipid bilayer (Fig. 2, lower panels). When current jumps of intermediate levels were rare or difficult to resolve, as was the case with Cry1Ac, R525D, and R525G, only the principal conducting states were considered for conductance determination, i.e., the largest detectable current steps for which direct transitions could be observed between the baseline and the conducting level. For the three other mutants, I-V curves were also obtained for the subconducting states in which the channel currents remained for significant periods of time. Such I-V curves were determined under 150/150 mM KCl (*cis/trans*) symmetrical ionic conditions for the wild-type toxin and all the mutants. Ion selectivities of Cry1Ac, R525G, R525G/R527G, and R529D were measured by conducting experiments under 450/150 mM KCl (*cis/trans*) nonsymmetrical conditions. In all cases, the I-V curves were rectilinear, which indicated that the channels did not rectify, i.e., that they passed current equally well in either direction.

In general, replacement of arginines by either a polar residue or a negatively charged residue did not significantly affect channel conductances (Table 3). Under asymmetrical ionic conditions, mutant conductances became larger compared to that of the same mutant under lower ionic conditions, and as with Cry1Ac, the channels were cation selective (Table 3). This was indicated by the fact that their current-voltage relations were shifted to the left, with zero-current voltages, i.e., reversal potentials, becoming more negative by approximately 21 to 27 mV (Fig. 2, lower panels). This shift was consistent with a Nernst equilibrium potential of −27.7 mV calculated for monovalent cations under these conditions (3:1 KCl concentration gradient).

However, for most mutants, qualitative differences were apparent in their membrane integration capability and channel kinetics (Table 3, last column). Whereas R525D and R525G channel activity was similar to that of Cry1Ac, partition of R525G into the lipid bilayer was more difficult: at an equivalent dose, it took longer for channel activity to be observed. Contrary to the case with Cry1Ac and R525D, R525G did not display fast flickering of channel current when negative voltage was applied. The channels formed by R527G displayed very low activity. They tended to remain closed or in a state in which conductance was about one quarter of the maximum conductance seen for this protein. The double mutant R525G/R527G also displayed low activity at positive voltages. At negative voltages, the channel currents were less stable and displayed significant flickering like those observed with R525D under the same conditions. Finally, the 529D mutant was also less active than the wild-type toxin and remained essentially locked in one of its subconducting states.

DISCUSSION

Sequence alignments of various Cry toxin classes have shown that the most conserved arginine in the alternating arginine region (bloc 4 in DIII) is localized to the fourth or most C-terminal position, followed by the third position, with the first and second positions showing the most variability (Table 2). In Cry1Aa, the second and third arginines are involved in hydrogen bonding to two main chain carbonyl oxygen atoms (R523) or in a salt bridge triad with two other charged residues (R600 and E602), suggesting that these residues as well as the fourth arginine, which also forms a salt bridge, are very important for structural stability (11). The first arginine position in all toxin classes examined (Table 2) can generally form hydrogen bonds and is accessible, unlike the third and fourth positions, to the solvent. In Cry1Ac, structural modeling also predicts that as with Cry1Aa, salt bridges can occur at the third and fourth positions. Among the single-residue mutants, the greatest decrease in toxicity occurred for those in which the first or third arginine (positively charged) was replaced by aspartic acid (negatively charged) rather than glycine (neutral). Why the first arginine position is the one most affected by mutation in in vitro bioassays but not in vivo bioassays is uncertain, but it is presumably due to the inherent differences between the two interspecific assay sytems. Indeed, the immediate question arising from the results derived from this study is whether mutations in these arginines play a direct role in ion channel function or an indirect role due to minor structural alterations causing a secondary effect, such as altered oligomerization in pore creation. Recently our laboratory and another group have presented data supporting an umbrella-like model for DI insertion into membranes (7, 19, 23). One consequence of this mode of insertion would be the approach of the β17 strand, and consequently the alternating arginines, nearer to the mouth of the pore. An increase or decrease in the number of positive charges near the pore mouth would presumably affect pore conductance. In relation to the first arginine position, loss of toxicity may be related to the exposure of this position, unlike the others, to the solvent. Unlike the case with previous studies, we do not see such an alteration in the rate of ion flow through the channel but rather see an increase in the length of time for channel activity to appear. The lower activity level of R526G suggests that membrane permeation was difficult to achieve, presumably due to either a structurally altered toxin or possibly difficulty in properly forming the oligomeric pore structure in the membrane (1).

In an earlier study, Chen et al. (4) were unable to produce protoxins mutated at the second or third arginine position. Considering that the β17 strand is highly similar between the two Cry1A toxins, the ability to express Cry1Ac mutants in the present study was probably due to the different nature of the amino acids substituted in these positions. Mutant toxins at these positions resembled wild-type Cry1Ac both biochemi-

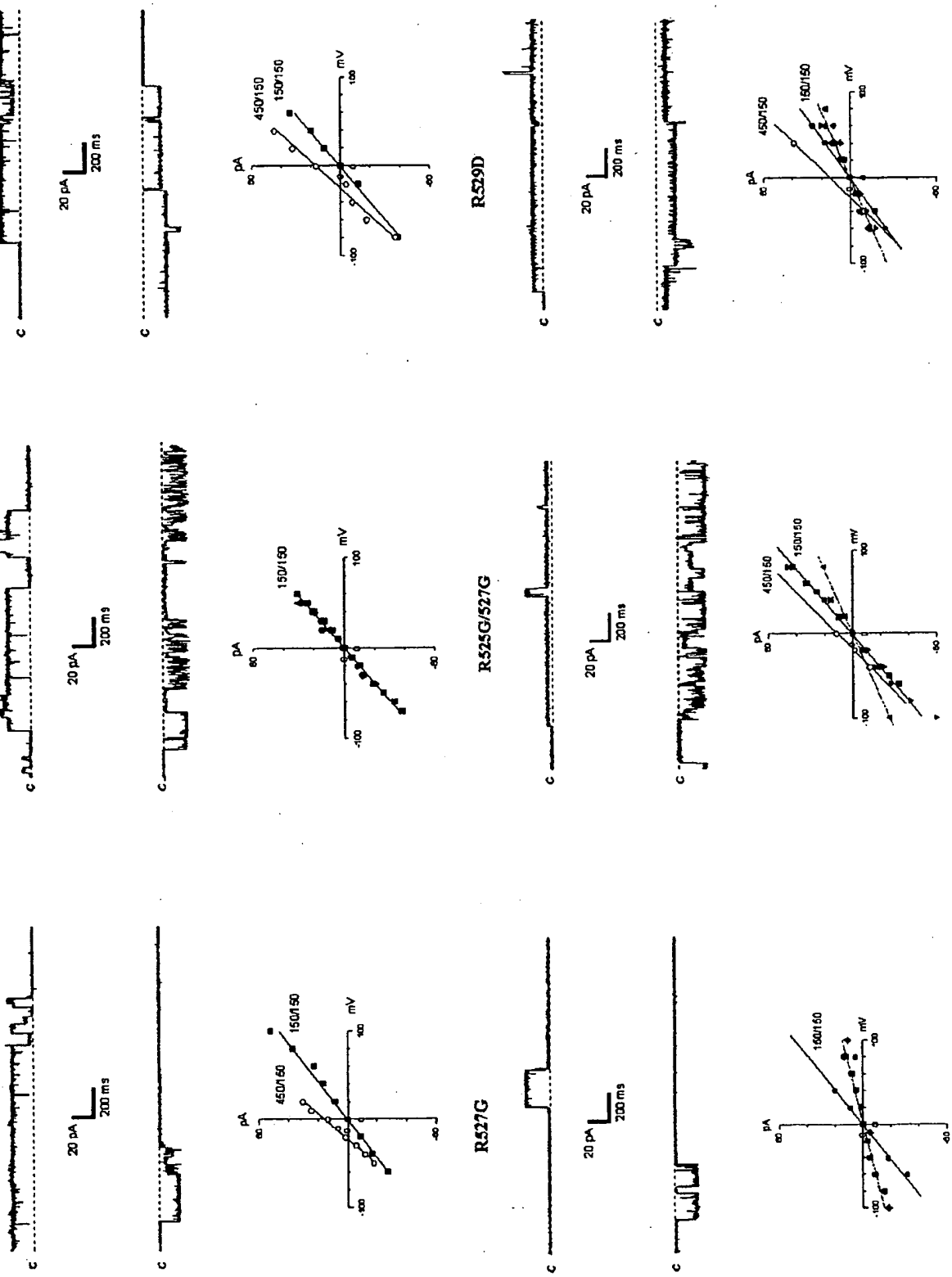

FIG. 2. Single-channel current traces. Representative single-channel current traces, recorded after the partition of Cry1Ac and the five arginine mutants into planar lipid bilayers separating symmetrical (150 mM *cis*/150 mM *trans*) KCl buffer solutions at pH 9.0 are shown in the upper traces (applied voltage, +40 mV), and middle traces (−40 mV). The letter *c* on the left of the traces indicates the closed state of the channels. Current-voltage relations (I-V curves) of the channels are shown directly under the single-channel current traces. Data points were fitted by linear regression.

TABLE 3. Characterization of ion channels by arginine replacement mutants in planar lipid bilayers

Toxin	Conductance[a,b] (pS)		Subconductance[a,c] (pS)	Selectivity (V_r)[f]	Activity level
	150/150 mM KCl	450/150 mM KCl	150/150 mM KCl		
Cry1Ac	457 ± 13 (3)	688 (1)	ND[d]	Cationic (22.5 mV)	Wild-type
R525D	522 ± 20 (3)	ND	ND	ND	Similar to wild-type
R525G	526 ± 52 (5)	732 ± 31 (3)	ND	Cationic (26.5 mV)	Similar to wild-type, but difficult to incorporate into PLBs[e]
R527G	500 (1)	ND	148 ± 10 (4)	ND	Very low; mutant tends to stay in its subconducting state
R529D	424 (1)	637 ± 27 (2)	213 ± 11 (4) 302 (1)	Cationic (24 mV)	Low; mutant tends to stay in one or more subconducting states
R525G/R527G	492 ± 65 (4)	590 (1)	86 (1)	Cationic (21 mV)	Low

[a] Mean ± standard error of the mean.
[b] Numbers in parentheses under conductance data indicate the number of experiments in which the principal conductance level could be observed and measured.
[c] Numbers in parentheses under subconductance data indicate the number of experiments in which subconducting states could be observed and reliably determined according to Fox (6).
[d] ND, not determined.
[e] PLBs, planar lipid bilayers.
[f] V_r is the reverse potential (zero-current potential) determined under 450/150 mM KCl (*cis*/*trans*) nonsymmetrical ionic conditions.

cally in their alkaline solubility and protease sensitivity and in bioassays using susceptible insect larvae or, with a few exceptions, in a highly sensitive in vitro lawn assay, on cultured insect cells. When the pore-forming characteristics of these exceptions (R525D, R525G, R527G, R529D, and R525G/R527G) showing slight toxicity differences were examined in planar lipid bilayers, it was determined that the two internal arginine mutants possessed a feature in addition to the low channel activity observed with R525G. Both R527G and R529D formed channels that displayed a tendency to remain in a subconducting state. These data support the notion that structural alterations in this region have an indirect affect on ion channel function, since different subconducting states are generally a reflection of the oligomerization of different pore subunits, or in this case toxin molecules. Either altered toxin oligomerization or more likely a slightly deformed pore may explain why the two internal arginine mutants are more often locked into a subconducting state.

Taken together, our results suggest that the primary role of this conserved β17-strand region is structural stability of the toxin. They also suggest that deviations in ion channel properties of mutant toxins in this region are an indirect consequence of structural alterations during or after membrane integration of the toxin, resulting in an improper pore architecture as manifested by slight differences in ion transport across the membrane.

ACKNOWLEDGMENTS

We thank A. M. Mes-Masson for a critical reading of the manuscript and Luc Péloquin, Naomi Finson, and Yong-Biao Liu for technical assistance.

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